Oncogene-induced senescence

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Oncogene-induced senescence: from *in vitro* tool to *in vivo* tumour suppression

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Oncogene-induced senescence: from *in vitro* tool to *in vivo* tumour suppression

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Liesbeth Cornelia Wilhelmina Vredeveld

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$BRAF^{E600}$ in benign and malignant human tumours

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BRAF^{E600} in benign and malignant human tumours

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ABSTRACT

Of the RAF family of protein kinases, BRAF is the only member to be frequently activated by mutation in cancer. A single amino acid substitution (V600E) accounts for the vast majority and results in constitutive activation of BRAF kinase function. Its expression is required to maintain the proliferative and oncogenic characteristics of BRAF^{E600}-expressing human tumour cells. Although BRAF^{E600} acts as an oncogene in the context of additional genetic lesions, in primary cells it appears to be associated rather with transient stimulation of proliferation. Eventually, BRAF^{E600} signalling triggers cell cycle arrest with the hallmarks of cellular senescence, as is illustrated by several recent studies in cultured cells, animal models and benign human lesions. In this review, we will discuss recent advances in our understanding of the role of BRAF^{E600} in benign and malignant human tumours and the implications for therapeutic intervention.

RAF KINASE FAMILY IN SIGNAL TRANSDUCTION

BRAF is a member of the RAF family of protein kinases, comprising three members: ARAF, BRAF and CRAF (reviewed in Chong et al., 2003). Homologues of the three corresponding genes are found in all vertebrates, while a single *Raf* gene exists in invertebrates (*e.g. D-Raf* in *Drosophila melanogaster* and *lin-45* in *Caenorhabditis elegans*), whose sequence is most closely related to BRAF. The *RAF* gene products are RAS effectors, participating in the ERK (MAPK) signalling pathway, which connects extracellular signals to transcriptional regulation (reviewed in McKay & Morrison, 2007) (Figure 1). RAS is activated by growth factor and hormone signalling and activates multiple downstream pathways controlling cellular survival, proliferation and differentiation. In its active GTP-bound state it binds to RAF proteins (also named MAPKKKs), thereby recruiting them to the plasma membrane. Once localised at the membrane these kinases are activated by a series of phosphorylation and

dephosphorylation events. BRAF and CRAF can also heterodimerise in response to mitogenic signals and the activity of the complex requires at least one of the two monomers to be active (Rushworth et al., 2006). Activated RAF proteins phosphorylate and activate MEK1 and 2 (MAPKKs), which in turn phosphorylate and activate ERK1 and 2 (MAPKs). These phosphorylate several cytoplasmic and nuclear targets, including a set of transcription factors such as Ets-1, c-Jun and c-Myc. The multiple steps in the RAS/RAF/MEK/ERK pathway provide a mechanism of signal amplification as well as a platform for signal modulation by other factors.

The first *RAF* member to be cloned was murine *Craf* (often referred to as *Raf-1*), which was identified as an oncogene carried by the 3611-MSV virus. It was

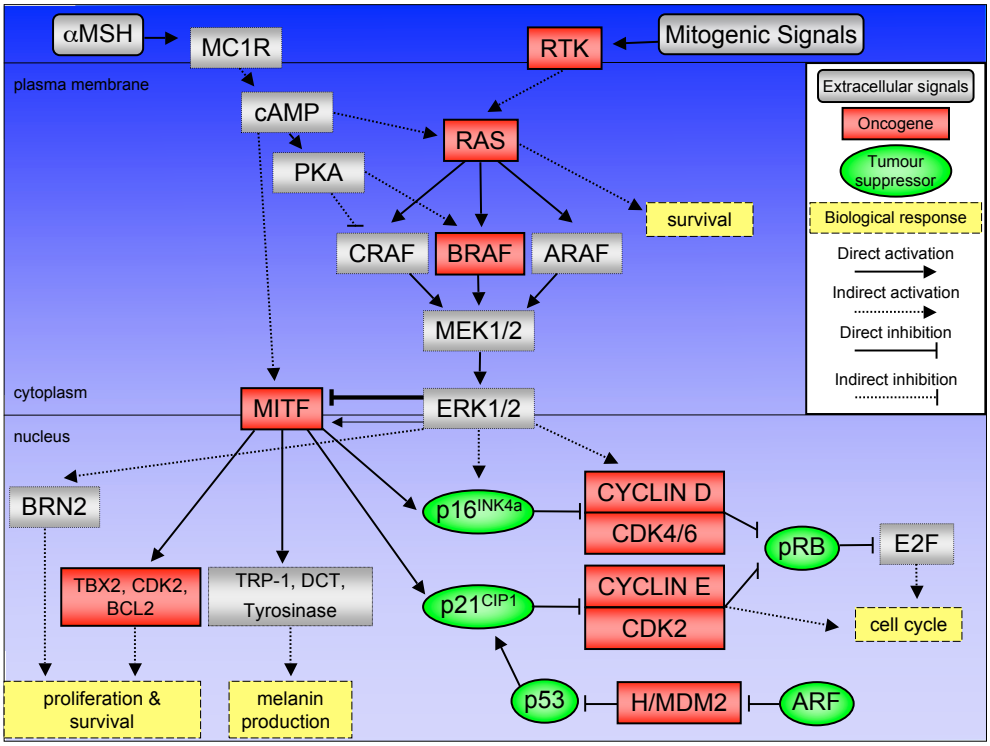

Figure 1 | ***Signalling pathways involved in tumourigenesis.*** Schematic representation of the RAS/RAF/MEK/ERK signalling pathway, which feeds into various effector processes, including those governing cell proliferation and survival. In addition, in melanocytes the MITF transcription factor is under (both positive and negative) control of BRAF- (and cAMP-) dependent signals to regulate melanin production in response to αMSH. Whereas in non-malignant cells BRAF activity is modulated as a function of extracellular signals through RTKs, the cancer-derived $BRAF^{E600}$ mutant functions autonomously. A central cell cycle pathway downstream of BRAF corresponds to the $p16^{INK4a}$/CDK4/pRB/E2F route, which in melanocytes is also under control of MITF. The CDK inhibitor $p21^{CIP1}$ acts as a nodal point connecting the pRB pathway to the p53 tumour suppressor and MITF. Proteins are colour-coded as explained in the insert.

designated v-*raf* because of the ability of the virus to induce Rapidly growing Fibrosarcomas in mice (Rapp et al., 1983). Around the same time, chicken *Raf* was cloned from an avian virus (Mill-Hill No 2) and termed v-*mil* (Jansen et al., 1984; Jansen et al., 1983). Cloning of human *CRAF*, *ARAF* and *BRAF* followed shortly afterwards (Bonner et al., 1985; Huebner et al., 1986; Beck et al., 1987; Ikawa et al., 1988; reviewed in Wellbrock et al., 2004). In 2003, an omission of three nucleotides from the first exon in the sequence of the human *BRAF* gene in NCBI was identified and corrected, resulting in the change of the numbering of protein residues (Kumar et al., 2003a). In this review we will be using the updated numbering system (reviewed in Wellbrock et al., 2004).

RAF proteins contain three Conserved Regions, CR1, 2 and 3 (Figure 2) (reviewed in Garnett & Marais, 2004; Gray-Schopfer et al., 2005). CR1 and CR2 are regulatory domains. Binding to RAS and recruitment to the plasma membrane is accomplished through the RAS-Binding Domain (RBD) and the Cystein-Rich Domain (CRD), both of which are located in CR1. CR3 comprises the kinase domain, which in turn contains two regions important for RAF activation: the activation segment and the Negatively-charged regulatory-region (N-region). Phosphorylation of two key residues (T^{599} and S^{602} for BRAF) within the activation segment is necessary for RAF activation (Zhang & Guan, 2000; reviewed in Gray-Schopfer et al., 2005). The N-region contains a SSYY motif (S^{338}SYY in CRAF and S^{298}SYY in ARAF), which is also subject to phosphorylation (Fabian et al., 1993; King et al., 1998). The first serine and last tyrosine residues of this motif in CRAF and ARAF must be phosphorylated for activation (Marais et al.,

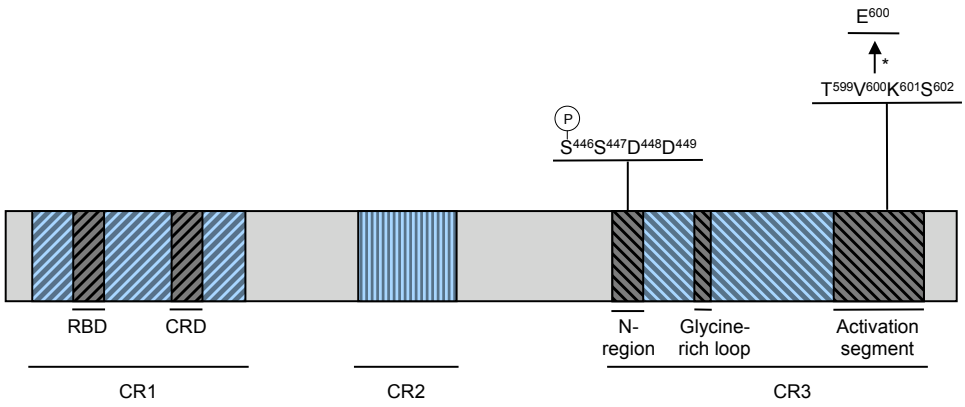

Figure 2 | ***Schematic representation of the BRAF protein kinase.*** The three Conserved Regions CR1, 2 and 3 are highlighted blue. CR1 and 2 are regulatory domains and CR3 represents the catalytic domain. The Ras Binding Domain (RBD) and Cystein Rich Domain (CRD) are located in CR1. The N-region, Glycine-rich loop and Activation segment are located in CR3. S^{446} in the SSDD motif, responsible for the negative charge of the N-region in BRAF, is constitutively phosphorylated. Phosphorylation of residues T^{599} and S^{602} results in BRAF activation. The position of the mutational hotspot V600E is indicated.

1997; Mason et al., 1999). In contrast, the serine residue S^{446} in BRAF is constitutively phosphorylated, and instead of tyrosine residues, aspartic acids ($D^{448}D^{449}$) are encoded, mimicking phosphorylated tyrosines (Figure 2). As a result, activation of BRAF requires fewer phosphorylation steps. These structural differences with the two other RAF family members, can explain the higher steady state kinase activity of BRAF (reviewed in Garnett & Marais, 2004; Gray-Schopfer et al., 2005), which is further supported by the finding that the N-region sequence and phosphorylation status play essential roles in determining the kinase activity of both BRAF and CRAF cancer-related mutants (Emuss et al., 2005).

BRAF MUTATIONS IN CANCER

BRAF is the only RAF protein to be frequently mutated in cancer, probably because its constitutive activation requires fewer mutational events. No *ARAF* mutations have been identified so far (Emuss et al., 2005; Lee et al., 2005). *CRAF* mutations are rarely found (Emuss et al., 2005; Zebisch et al., 2006), but the gene is overexpressed in some ovarian and pulmonary carcinomas, raising the possibility that also *CRAF* can act as an oncogene in man (McPhillips et al., 2006; Rapp et al., 1988).

Biochemical characteristics of cancer-associated BRAF mutants

The identification of *BRAF* mutations in human cancers stimulated intensive study of this gene (Davies et al., 2002). Mutations were identified in approximately 66% of melanomas, and in a smaller percentage of other tumours, including thyroid, colonic and ovarian carcinomas and some sarcomas (Davies et al., 2002; Cohen et al., 2003; Kimura et al., 2003). The most common *BRAF* mutation corresponds to a T>A transversion at position 1799, resulting in the substitution of Valine by Glutamate at position 600 of the protein (Figure 2). The mutant amino acid is situated between residues T^{599} and S^{602}, the phosphorylation of which is responsible and sufficient for BRAF activity. The V600E mutation is therefore thought to mimic T^{599}/S^{602} phosphorylation, rendering BRAF constitutively active (Figure 3). This view is further supported by the finding that $BRAF^{V600E}$ (from hereon referred to as $BRAF^{E600}$) displays increased kinase activity relative to the wild type (wt) protein and has transforming capacity (Davies et al., 2002). Furthermore, $BRAF^{E600}$ is insensitive to the SPRY2-mediated negative feedback loop, which inhibits MEK/ERK signalling in cells expressing wt BRAF (Tsavachidou et al., 2004). $BRAF^{E600}$ activity is also independent of the presence of a negative charge in the N-region normally required for wt BRAF and CRAF activation (Emuss et al., 2005; Brummer et al., 2006).

Cell lines expressing $BRAF^{E600}$ are dependent on it for proliferation and survival (Hingorani et al., 2003; Calipel et al., 2003; see below) and most do not require RAS for proliferation (Davies et al., 2002), although some are sensitive to RAS inhibition

in an ERK-independent way (Calipel et al., 2003) (Figure 3). The capacity of $BRAF^{E600}$ to signal independently from RAS is further supported by the observation that chicken $BRAF^{E600}$ harbouring a second mutation in the RBD that prevents RAS binding, can still induce ERK phosphorylation (Brummer et al., 2006). Intriguingly, although most BRAF mutants display elevated kinase activity compared to the wt protein, four cancer-derived mutants have reduced kinase activity (Wan et al., 2004). Three of these mutants ($BRAF^{G466E}$, $BRAF^{G466V}$ and $BRAF^{G596R}$) are capable of inducing ERK phosphorylation through heterodimerisation with CRAF (Figure 3). The fourth mutant ($BRAF^{D594V}$) acts like a kinase-dead mutant and cannot bind CRAF. Its role in tumourigenesis remains to be elucidated. Although all BRAF cancer mutants are capable of dimerising with CRAF, only the three 'impaired kinase activity' mutants rely totally on CRAF for ERK activation (Wan et al., 2004).

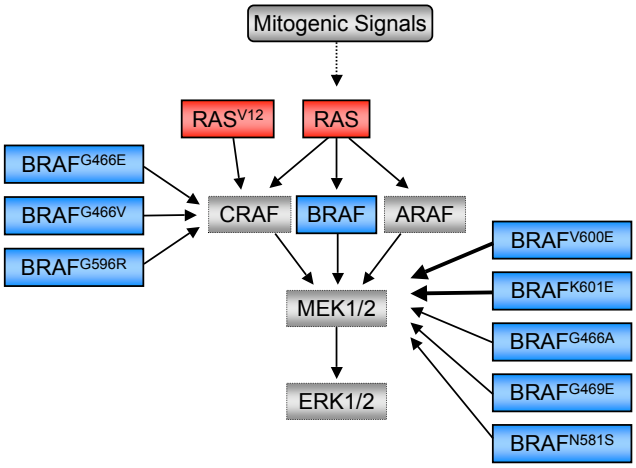

Figure 3 | ***Oncogenic signalling by the RAS/RAF pathway.*** In response to mitogenic stimuli, wild type RAS can signal to all three RAF proteins. Oncogenic RAS and BRAF mutants activate MEK/ERK independently of mitogenic signals. In melanoma cell lines, RAS^{V12} signals exclusively via CRAF. BRAF mutants associated with high kinase activity (block arrows; e.g. V600E, K601E, or similar mutants (not shown)) or intermediate activity (thin arrows; e.g. G466A, G469E, N581S, or similar mutants (not shown)) stimulate MEK directly, whereas impaired activity mutants (G466E, G466V and G596R) require CRAF for MEK activation.

Melanoma susceptibility and BRAF mutations

Germline *BRAF* mutations have recently been identified in Cardio-Facio-Cutaneous (CFC) syndrome patients. From a total of 12 different mutations, 5 are found in codons mutated in cancer of which 2 correspond to cancer-related substitutions. However, the association of CFC syndrome with cancer is rare (Rodriguez-Viciana et al., 2006; Niihori et al., 2006; van Den Berg & Hennekam, 1999). Two *BRAF* mutations

have also been identified in individuals with Costello syndrome (CS), a syndrome similar to the CFC (Rauen, 2006). No $BRAF^{E600}$ mutations have been identified in either CFC syndrome or CS patients so far. Studies of melanoma patients have revealed little evidence that *BRAF* is a melanoma susceptibility gene (Meyer et al., 2003a; Laud et al., 2003; Lang et al., 2003; Jackson et al., 2005). There is only one report on two germline mutations (M116R and Q608H) in three individuals (Casula et al., 2004). In a $BRAF^{E600}$ knock-in mouse model, expression of $BRAF^{E600}$ in all tissues results in embryonic lethality by day E7.5, making it unlikely that this mutation would be compatible with life in humans (Mercer et al., 2005). It is, however, intriguing that some CFC mutants display apparently similar *in vitro* kinase activity as $BRAF^{E600}$. The possible contribution of *BRAF* single nucleotide polymorphisms (SNPs) in melanoma susceptibility remains a controversial issue. Non-coding SNPs in the *BRAF* gene have been linked with increased risk of melanoma development (Meyer et al., 2003b; James et al., 2005), although opposing results have also been published (Laud et al., 2003; Jackson et al., 2005).

BRAF mutations in malignant tumours

Among human cancers, *BRAF* mutations are most common in melanoma. The prevalence of *BRAF* mutations varies substantially amongst the different types of melanoma. They are most common in cutaneous melanoma, rare in mucosal, acral and conjunctival melanomas, and virtually absent from uveal melanomas. So, although melanoma can arise in all of the locations where melanocytes reside, oncogenic *BRAF* mutations are most frequently observed in melanomas that arise in the skin. Furthermore, within the group of cutaneous melanomas, *BRAF* mutations are found primarily in melanomas of intermittently sun-exposed skin rather than chronically sun-damaged skin. The latter melanomas, which typically harbour wt BRAF, generally arise later in life (Maldonado et al., 2003; Cohen et al., 2004; Curtin et al., 2005). Mutations in the RAS/RAF/MEK/ERK pathway components are frequently observed in melanoma; in cases where *BRAF* is not mutated, other oncogenic lesions affect proteins acting either upstream or downstream of BRAF (Chin et al., 2006). *NRAS* mutations are found in all subtypes of melanoma, although the overall mutation frequency is lower than that of *BRAF* (*e.g.* 22% *versus* 59% in the study by Curtin *et al.*, 2005). With few exceptions, *BRAF* and (*N-, H-, K-*) *RAS* mutations are mutually exclusive. Although there are some reports of *BRAF* mutations coinciding with *RAS* mutations within the same tumour, the oncogenic nature of these mutations and their co-existence in the same cell are not always clear (Davies et al., 2002; Gorden et al., 2003; Kumar et al., 2003b; Sensi et al., 2006). Mutations in *cKIT*, a tyrosine kinase receptor for Kit ligand (Stem Cell Factor (SCF)), are most common in mucosal, acral and chronically sun damaged skin melanomas (Curtin et al., 2006; Willmore-Payne et al., 2005; Willmore-Payne et al., 2006). *CCND1*, encoding Cyclin

D1, is amplified most commonly in melanomas without *BRAF* or *RAS* mutations, whereas melanomas with mutation in either of the latter two genes display elevated Cyclin D1 levels (Errico et al., 2003; Curtin et al., 2005). *CDK4* amplifications are more common in acral and mucosal melanomas and are also inversely correlated with *BRAF* or *RAS* mutations or *CCND1* amplifications (Curtin et al., 2005). It is therefore evident that constitutive activation of the RAS/MEK/ERK pathway, by oncogenic lesions in one of the pathway's components, is an important step in melanoma development.

In addition to genetic alterations in *RET/PTC*, *NTRK1*, *PPARγ*, *HRAS* and *NRAS*, mutations in *BRAF* represent the most common oncogenic lesion found in thyroid cancer (reviewed in Xing, 2005; Ciampi & Nikiforov, 2005; Kondo et al., 2006; Trovisco et al., 2006). As in melanoma, *BRAF* mutations are mutually exclusive with *RAS* mutations or *RET/PTC* rearrangements: the presence of one seems to make the others redundant, probably because all result in activation of the MEK/ERK pathway. The V600E amino acid substitution accounts for virtually all BRAF mutations found in thyroid cancer (reviewed in Xing, 2005; Ciampi & Nikiforov, 2005; Kondo et al., 2006; Trovisco et al., 2006), with only one rare exception (K601E; Soares et al., 2003; Lima et al., 2004). $BRAF^{E600}$ is most common in papillary and anaplastic thyroid cancer (PTC (44%) and ATC (24%), respectively; Xing, 2005) and it is practically absent from follicular thyroid carcinomas and follicular adenomas (FTC and FTA respectively), which more often carry *RAS* mutations, and from medullary thyroid carcinomas (MTC). Within the PTC group, $BRAF^{E600}$ is more commonly found in the conventional PTC and tall-cell PTC subtypes, compared to follicular-variant PTC (reviewed in Xing, 2005; Ciampi & Nikiforov, 2005; Kondo et al., 2006; Trovisco et al., 2006). Furthermore, $BRAF^{E600}$ is observed in adult onset PTC as opposed to the rare paediatric PTC, which more often harbours *RET/PTC* rearrangements. However, a rearrangement of chromosome 7q involving *BRAF* and *AKAP9* is found in a subset of thyroid tumours in children exposed to radiation after the Chernobyl accident (Ciampi et al., 2005a). The chimeric protein displays similar kinase activity and transforming capacity as $BRAF^{E600}$, which probably accounts for its oncogenic role. Finally, gains of chromosome 7 or *BRAF* amplification have been reported in PTC, FTC and FTA, indicating a third possible mechanism of increased BRAF activity in thyroid tumours (Ciampi et al., 2005b).

Despite several attempts (Pavey et al., 2004; Bloethner et al., 2005), microarray gene expression analysis of large panels of melanoma cell lines has failed to reveal a significant and specific $BRAF^{E600}$ signature (Hoek et al., 2006). In contrast, signatures of different tumour subsets have been reported in melanoma specimens, for instance, radial *versus* vertical growth phase tumours (Haqq et al., 2005). Furthermore, a global expression analysis of a large panel of PTCs did reveal three

distinct gene expression profiles for tumours with *BRAF*, *RET/PTC* or *RAS* mutations, which correlate with histological tumour type (Giordano et al., 2005). However, it is still unclear whether specific mutations induce a specific type of tumour, or whether specific mutations confer a selective advantage to the tumour cells of some histological types, but not others. Perhaps the lack of a specific $BRAF^{E600}$ signature in melanoma cell lines reflects the fact that cell lines and not tumours were used for analysis.

A question arising from the analyses of melanoma and thyroid cancer mutations is why BRAF is more commonly activated in these tumours than RAS is. While BRAF is merely a RAS effector, activation of the latter is not just pro-mitogenic, via RAF/MEK/ERK signalling, but also increases cell survival through other pathways (Figure 1). The answer might lie in the nature of melanocytes and thyroid cells, as has been suggested recently by Marais and colleagues (Dumaz et al., 2006; Dhomen & Marais, 2007). Melanin production, one of the main functions of melanocytes, is stimulated by α-melanocyte stimulating hormone (αMSH) (Figure 1). This hormone is secreted by keratinocytes and binds to the melanocortin 1 receptor, MC1R (Sturm, 2002; Wong & Rees, 2005). This G protein-coupled receptor triggers the cyclic adenosine monophosphate (cAMP) pathway in a Protein Kinase A (PKA)-dependent fashion. Similarly, in thyroid follicular cells, thyroid stimulating hormone (TSH) signalling results in elevated cAMP levels. This second messenger can either activate or suppress the ERK pathway and can have a pro- or anti-proliferative effect, depending on cellular context. This dual potential seems to depend partially on the RAF kinase in use (Stork & Schmitt, 2002). When present, BRAF seems to be the preferred MAPKKK and as such is most often activated by cAMP signalling. In BRAF-expressing cells of melanocytic origin (normal human melanocytes, immortal mouse melanocytes and mouse melanoma cells), in which cAMP plays a key role in proliferation and differentiation, cAMP activates the ERK pathway in a RAS- and BRAF-dependent way (Busca et al., 2000; Dumaz et al., 2006). cAMP signalling also leads to PKA-dependent CRAF phosphorylation, rendering it incapable of RAS binding and thus inactive (Stork & Schmitt, 2002; Dumaz et al., 2006) (Figure 1). For this reason, it is likely that melanocytes use BRAF as the main RAS effector, similarly to melanoma cell lines harbouring mutant BRAF. When RAS is mutated however, BRAF is no longer necessary for ERK activation, whereas CRAF is (Dumaz et al., 2006) (Figure 3). Such a RAS-CRAF cascade would require inactive PKA and hence disrupted MC1R signalling. In accordance with this, melanoma cell lines carrying a RAS mutation are unresponsive to αMSH (Dumaz et al., 2006). It has therefore been suggested that RAS mutations become oncogenic in melanocytes only after a second hit that disrupts αMSH/MC1R signalling. Consequently, activating RAS mutations correspond to relatively rare events, compared to BRAF mutations, which can be tolerated in the

presence of functional MC1R signalling (Dhomen & Marais, 2007). The matters are more complicated however, as within the group of intermittently sun-exposed skin, *BRAF*-mutant tumours are often associated with the presence of one or more *MC1R* allele variants with reduced signalling capacity (Landi et al., 2006). In fact, individuals carrying two wt *MC1R* alleles are less likely to develop a mutant BRAF melanoma on intermittently sun-exposed skin, suggesting that disruption of MC1R signalling provides an advantage to $BRAF^{E600}$-expressing melanocytes, or might predispose them to the acquisition of the mutation. Hypofunctional MC1R variants however, could be associated with higher rates of mutation in general and not only $BRAF^{E600}$. The validation of this hypothesis will require analysis of a larger number of melanomas and melanoma cell lines with respect to *MC1R* allelic variants and αMSH responsiveness in the context of *RAS* and *BRAF* mutations.

BRAF mutations and UV radiation

Epidermal melanocytes are very near the skin surface and are thus relatively exposed to environmental mutagenic influences, especially UV irradiation (Miyamura et al., 2007). Melanocytes produce melanins, a family of closely related molecules derived from tyrosine, some (eumelanins especially) providing protection from the damaging effects of solar radiation, but also constituting a risk of UV-induced macromolecular damage (mostly pheomelanins), since their irradiation results in formation of oxygen radicals. Given the established involvement of UV exposure in melanoma development, a possible explanation for the high prevalence of $BRAF^{T1799A}$ in melanoma would be that the mutation is UV-induced. However, the T>A transversion of this mutation is not a typical UV-induced lesion (C>T or CC>TT), which makes this scenario unlikely. Others have proposed that the establishment of the $BRAF^{T1799A}$ mutation occurs as a function of neighbouring UV-induced pyrimidine dimers (Thomas et al., 2006). According to another hypothesis, the *BRAF* mutation might be the result of UV-induced oxidation of melanin, resulting in the formation of reactive oxygen species and leading to increased DNA-damage (Meyskens et al., 2001). As none of the above hypotheses has yet been proven, the mechanism -or mechanisms- underlying the acquisition of the $BRAF^{T1799A}$ mutation remains elusive. The fact that it is found in congenital melanocytic naevi (see below) that arise *in utero*, as well as in thyroid, colorectal and ovarian tumours, indicates that it can arise in the total absence of UV exposure. Whether it can occur also as a result of UV-radiation, possibly indirectly, remains to be proven.

The oncogenic function of $BRAF^{E600}$ in vitro and in xenograft models

Several laboratories have investigated the potential of $BRAF^{E600}$ to act as an oncogene. $BRAF^{E600}$ can transform NIH3T3 immortal fibroblasts, although less efficiently so than $HRAS^{V12}$ (Davies et al., 2002). Wellbrock *et al.* (2004) performed

experiments with mutant BRAF in an immortal mouse melanocyte cell line (melan-a cells) lacking expression of $p16^{INK4a}$ and ARF, two tumour suppressor proteins (see below) (Sviderskaya et al., 2002). Overexpression of $BRAF^{E600}$, but not of wt BRAF, induces constitutive MEK/ERK signalling and proliferation in the absence of TPA. Like RAS^{V12} (Wilson et al., 1989) and a constitutive active MEK mutant (MEK^{EE}), $BRAF^{E600}$-expressing melan-a cells grow anchorage-independently and give rise to tumours when injected subcutaneously (s.c.) in immunodeficient mice. $BRAF^{E600}$ overexpression in another immortal but well-differentiated rat cell line of thyroid origin (PCCL3 cells), induces DNA synthesis, but also apoptosis. Thyroid-specific differentiation genes are downregulated and chromosomal instability is induced (Mitsutake et al., 2005). Related studies demonstrate a transformed phenotype of these cells through the ability to invade matrigel, due to upregulation of several matrix metalloproteinases (MMPs) (Mesa et al., 2006). $BRAF^{E600}$ can also transform human diploid fibroblasts (HDFs) in the context of a defined set of genetic lesions namely, hTERT, SV40 small t (st) and disruption of the pRB and p53 pathways, as shown previously for RAS^{V12} (Michaloglou et al., 2005; Hahn et al., 2002). Thus, at least in the context of immortalizing genetic lesions, $BRAF^{E600}$ can contribute to oncogenic transformation of cultured cells.

Several studies investigating the role of $BRAF^{E600}$ on proliferation and survival of melanoma cells lines have provided further insight into its role in carcinogenesis. Treatment of cultured melanoma and thyroid cancer cell lines with either small interfering RNA (si-RNA, inducing transient silencing; Calipel et al., 2003; Salvatore et al., 2006) or short hairpin RNA (sh-RNA, mediating stable silencing; Hingorani et al., 2003) targeting mutant or both wt and mutant BRAF, leads to reduced phosphorylation of MEK and ERK (p-MEK and p-ERK), induction of cell cycle arrest, loss of anchorage independency and, depending on the cell line, induction of apoptosis. The growth arrest triggered by $BRAF^{E600}$ depletion is accompanied by reduction of cyclin D1 and D3, established RAS/MEK/ERK effector proteins, which is associated with an accumulation of the hypo-phosphorylated (activated) form of pRB (Rotolo et al., 2005). Also other downstream effectors of the RAS/MEK/ERK pathway, including BRN2 and MITF, are regulated by $BRAF^{E600}$ expression (Figure 1). BRN2 is a transcription factor often overexpressed in melanoma (reviewed in Vance & Goding, 2004). Loss of $BRAF^{E600}$ expression reduces BRN2 levels, and downregulation of BRN2 leads to decreased proliferation of melanoma cell lines (Goodall et al., 2004). In contrast, in some of the melanoma cell lines studied silencing of $BRAF^{E600}$ expression results in stabilisation of MITF (a key transcription factor in melanocyte biology; see below), leading to upregulation of tyrosinase and the tyrosinase-related protein 1 (TRP-1). This induces melanin production and maturation of melanosomes and subsequently stimulates pigmentation (Rotolo et al., 2005). Although in many human

tumours, the degree of differentiation is linked to prognosis, increased melanoma pigmentation does not correlate with decreased aggressiveness. In addition to being required for melanoma cell proliferation and survival, $BRAF^{E600}$ has been shown to contribute to invasion. Its silencing inhibits matrigel invasion, which is accompanied by a reduction of MMP2 activity and a decline in β_1-integrin protein levels in melanoma cells (Sumimoto et al., 2004), consistent with $BRAF^{E600}$ overexpression studies promoting invasion (Mesa et al., 2006).

The proliferative activity of melanoma cell lines *in vivo*, is also highly dependent on $BRAF^{E600}$. Immunodeficient mice injected s.c. with melanoma cells expressing sh-RNAs targeting BRAF develop smaller tumours with fewer cycling cells than control injected mice (Sumimoto et al., 2004). Likewise, experiments with inducible sh-BRAF show that $BRAF^{E600}$ silencing in an established tumour (100 mm^3) inhibits further tumour progression (Hoeflich et al., 2006). In some cell lines, $BRAF^{E600}$ silencing even results in complete tumour regression. This effect is caused by loss of proliferation, increased apoptosis and macrophage infiltration. Although even large tumours (1500 mm^3) can regress upon $BRAF^{E600}$ silencing, its reactivation results in rapid tumour relapse (Hoeflich et al., 2006). All of these studies indicate that melanoma cell lines carrying mutant BRAF are addicted to it. 'Oncogene addiction', which denotes the dependency of the cancer cell on the mutated oncogene (or inactivated tumour suppressor gene) (Weinstein, 2002), is observed for numerous cancer-associated genes, including NRAS in melanoma cell lines (Eskandarpour et al., 2005), and can probably be exploited clinically, making $BRAF^{E600}$ a promising drug target (see below).

$BRAF^{E600}$ AND SENESCENCE OF PRIMARY HUMAN CELLS

Replicative senescence denotes the phenomenon of irreversible proliferative arrest, first described for cells aged in culture (Hayflick, 1965) and more recently also *in vivo* (Herbig et al., 2006). Primary human cells can undergo several 'mortality' (M) stages of senescence. M0 is due to stress related with culturing conditions (*e.g.* exposure to supraphysiologic oxygen levels). M1 denotes arrest resulting from a telomere erosion-induced DNA damage response (Hayflick limit). M2 is caused by extreme telomere attrition, leading to chromosomal fusions and cell death. The critical role of telomeres in cancer is illustrated, for example, by the observation that mice deficient for the RNA component of telomerase ($mTR^{-/-}$) are resistant to MYC-driven lymphomagenesis: the incipient tumour cells become senescent in a p53-dependent manner (Feldser & Greider, 2007). In most cell types *in vitro*, the different mortality stages can be prevented or bypassed by disruption of the $p16^{INK4a}$/pRB and/or p53 pathways and/or ectopic expression of the catalytic subunit of telomerase (hTERT) (reviewed in Bennett & Medrano, 2002; Bennett, 2003; Shay & Wright, 2005). M0/1

senescent cells typically are large and flat, remain alive and metabolically active, but have lost responsiveness to growth factors (Hayflick, 1965). Senescent cells commonly display increased Senescence-Associated-β-Galactosidase (SA-β-Gal) activity (Dimri et al., 1995) and elevated PAI-1 levels (Goldstein et al., 1994). They also form Senescence-Associated Heterochromatic Foci (SAHF), stable pRB-dependent heterochromatin structures that repress E2F target genes, therefore contributing to the irreversibility of senescence (Narita et al., 2003). Senescence can be induced, also prematurely (i.e., in the absence of telomere attrition), by a variety of stressful conditions such as tissue culture stress, DNA damage, oncogene activation and cytotoxic drugs.

More than two decades ago, it was observed that mutationally activated RAS, introduced in untransformed fibroblasts, induced cell cycle arrest rather than oncogenic transformation (Land et al., 1983; Franza et al., 1986). Only in the context of certain oncogenic mutations did RAS contribute to oncogenic transformation (Land et al., 1983). In 1997, Serrano *et al*. were the first to describe the mechanism underlying this phenomenon of 'oncogene-induced senescence'. $HRAS^{V12}$ was demonstrated to induce a permanent G1 cell cycle arrest in primary fibroblasts of murine (MEFs) or human (HDFs) origin (Serrano et al., 1997). Oncogene-induced senescence is not restricted to fibroblasts but is observed in other cell types, including primary rat Schwann cells (Lloyd et al., 1997), human ovarian surface epithelial cells (Nicke et al., 2005) and primary human melanocytes (Denoyelle et al., 2006). As has become obvious from many subsequent studies, cells undergoing oncogene-induced senescence share the key features and cellular markers with cells undergoing replicative senescence: total loss of proliferative activity, a flat and enlarged cellular morphology, SAHF formation, increased SA-β-GAL activity and elevated PAI-1 levels. Importantly, oncogene-induced senescence is accompanied by the activation or induction of several tumour suppressors, including $p16^{INK4a}$, ARF, p53 and $p21^{CIP1}$ (Serrano et al., 1997). $p16^{INK4a}$ and ARF are both encoded by the *CDKN2A* locus (reviewed in Sherr, 2001). $p16^{INK4a}$ inhibits Cyclin Dependent Kinases 4 and 6 (CDK4/6), thereby causing pRB to accumulate in its hypophosphorylated form. In this way, pRB acquires increased affinity for, and inhibits E2Fs, transcription factors involved in cell cycle progression, DNA replication and repair. ARF inhibits the ubiquitin ligase HDM2 (MDM2 in mouse), resulting in p53 stabilization (Figure 1). The induction of tumour suppressors is one of the hallmarks of senescence, which distinguishes it from normal cellular differentiation, which is thought to depend on physiological cues, rather than oncogene activation.

ΔRAF1-ER, $BRAF^{E600}$, MEK^{P56}, all downstream and constitutively active effectors of RAS, also induce premature senescence in HDFs (Zhu et al., 1998; Michaloglou et al., 2005; Lin et al., 1998) and primary human melanocytes (Michaloglou et

al., 2005; Denoyelle et al., 2006; Gray-Schopfer et al., 2006). Inhibition of the MEK/ERK pathway can prevent $HRAS^{V12}$-induced senescence (Zhu et al., 1998), illustrating the dependency of RAS^{V12}-induced senescence on the RAF/MEK/ERK pathway. These cells also display several senescence markers, but become small and refractile instead of adopting large and flat morphology and they do not arrest strictly in G1, but also in G2/M. These differences might be because in addition to the MEK/ERK pathway, RAS can activate other signal transducers, including PI3K, RAL-GDS and the Rho family of small GTPases. Moreover, upregulation of p53 and $p21^{CIP1}$ is not detected in $BRAF^{E600}$-induced senescent cells, but this could be cell line-dependent (Zhu et al., 1998; Lin et al., 1998; Michaloglou et al., 2005). Primary human melanocytes that overexpress $BRAF^{E600}$ show a transient and moderate increase in proliferative activity prior to entering senescence (Michaloglou et al., 2005). This contrasts the more rapid cell cycle arrest mediated by $HRAS^{V12}$, which is accompanied by massive vacuolization and expansion of the endoplasmatic reticulum (ER) as part of the unfolded protein response (UPR). This response is critically involved in mediating $HRAS^{V12}$-induced senescence in melanocytes and acts independently of p53 and $p16^{INK4a}$ (Denoyelle et al., 2006). In knockin MEFs, $BRAF^{E600}$ fails to induce senescence, although the presence of additional genetic lesions has not been excluded (Mercer et al., 2005). In conclusion, in many, but not all (Benanti & Galloway, 2004), systems tested, ectopic expression of constitutively active members of the RAS/RAF/MEK/ERK signalling pathway induces premature senescence *in vitro*.

Although both $BRAF^{E600}$ and RAS^{V12} induce $p16^{INK4a}$, the subsequent premature senescence cannot be prevented by $p16^{INK4a}$ knockdown, at least in cultured human cells (Voorhoeve & Agami, 2003; Michaloglou et al., 2005; Denoyelle et al., 2006). In contrast, human fibroblasts harbouring a small homozygous germline deletion in $p16^{INK4a}$ are resistant to RAS^{V12}-induced senescence, but still enter senescence induced by $BRAF^{E600}$ (Brookes et al., 2002; Michaloglou et al., 2005). Neither RAS^{V12} nor $BRAF^{E600}$ induces ARF expression in human cells (Wei et al., 2001; Michaloglou et al., 2005). $p21^{CIP1}$, a key transcriptional target of p53, is not induced by $BRAF^{E600}$ (Gray-Schopfer et al., 2006), and consistently, p53 knockdown is not sufficient to prevent $HRAS^{V12}$- or $BRAF^{E600}$-induced senescence (Denoyelle et al., 2006).

$BRAF^{E600}$ AND ONCOGENIC TRANSFORMATION OF PRIMARY HUMAN MELANOCYTES

Chudnovsky *et al.* (2005) used reconstituted human skin grafts containing transformed human melanocytes on immunodeficient mice to study the oncogenic potential of a number of melanoma-associated genetic lesions, including $BRAF^{E600}$. When primary human melanocytes, transformed with hTERT, a dominant negative

p53 mutant ($p53^{DN}$), a $p16^{INK4a}$-insentive CDK4 mutant ($CDK4^{R24C}$) and $NRAS^{V12}$, are co-cultured with human keratinocytes on artificial human dermis and grafted onto immunodeficient mice, they form invasive tumours. Whereas activated PI3K can effectively replace $NRAS^{V12}$, $BRAF^{E600}$-transformed melanocytes unexpectedly induce only mild junctional hyperplasia, even though ERK signalling is activated to the same extent as in $NRAS^{V12}$-expressing cells. In this particular experimental system, PI3K pathway activation apparently represents a more potent oncogenic event than MEK/ERK pathway activation (Chudnovsky et al., 2005). Since $BRAF^{E600}$ contributes to melanoma cell proliferation and survival (Calipel et al., 2003; Hingorani et al., 2003), these results could be explained by assuming that in human melanocytes, $BRAF^{E600}$ acts oncogenically only in the context of other additional genetic lesions.

One such factor that might play a role in the proliferative outcome of $BRAF^{E600}$ signalling is the Microphthalmia-associated transcription factor (MITF), a melanocyte master regulator that is essential for melanocyte survival, maintenance, differentiation and pigmentation (reviewed in Goding, 2000) (Figure 1). One mechanism by which MITF is regulated is through phosphorylation by ERK, which leads to its activation, but at the same time to its degradation. In most melanoma cell lines the MEK/ERK pathway is constitutively active, resulting in low but persistent MITF levels. Since MITF is involved in cellular differentiation but also survival, it can act as both an oncogene and a tumour suppressor gene. Melanocyte cell lines with activated MEK/ERK signalling, for example as a result of $BRAF^{E600}$ overexpression, downregulate MITF and produce less pigment (Wellbrock & Marais, 2005). The reverse occurs when $BRAF^{E600}$ is downregulated in melanoma cell lines (Rotolo et al., 2005; see also above). Loss of MITF expression in human melanocytes results in downregulation of $p16^{INK4a}$ and $p21^{CIP1}$, as well as in hyperphosphorylation of pRB (Loercher et al., 2005; Carreira et al., 2005). Conversely, overexpression of MITF in non-transformed cells induces cell cycle arrest, which is accompanied by the activation of the genes encoding $p16^{INK4a}$ and/or $p21^{CIP1}$, corresponding to direct MITF transcriptional targets (Figure 1). However, when MITF is overexpressed in combination with $BRAF^{E600}$, it drives human melanocytes expressing hTERT/$CDK4^{R24C}$/$p53^{DN}$ to undergo oncogenic transformation. Consistent with a pro-oncogenic role for MITF in certain settings, *MITF* amplifications have been identified in melanoma, often in conjunction with mutant BRAF and inactivation of the $p16^{INK4a}$ pathway. Inactivation of MITF renders melanoma cell lines sensitive to cytotoxic drugs (Garraway et al., 2005). It appears that MITF can be added to a list of dual function cancer genes with context-dependent functions (Rowland & Peeper, 2006): it can act as a tumour suppressor by inducing antiproliferative proteins such as $p16^{INK4a}$ and $p21^{CIP1}$, and as an oncogene by stimulating proliferation- and survival-promoting genes (including BCL2, TBX2 and CDK2) (reviewed in Levy et al., 2006) (Figure 1).

Therefore, the biological effect of MITF activity is dependent on its genetic context, for example loss of specific tumour suppressor genes, but also on MITF levels and post-transcriptional modification (reviewed in Gray-Schopfer et al., 2007; Levy et al., 2006).

Weinberg and colleagues (Gupta et al., 2005) subjected primary human melanocytes to oncogenic transformation by ectopic expression of hTERT, SV40ER (st and LT) and RAS^{V12}. Similar to HDFs and human mammary epithelial cells (HMECs) that express these genes, these melanocytes grow anchorage-independently and form tumours when injected s.c. in immunodeficient mice. However, whereas mice injected with transformed HDFs and HMECs rarely develop metastases, all mice injected with transformed melanocytes rapidly developed metastases in the lung and to lesser extent in lymph nodes, liver, spleen and small bowel. This resembles the propensity of human melanomas to metastasize widely, even to distant sites such as the small bowel, which uncommonly harbours metastases of other tumour types. Indeed, human melanoma is an aggressive cancer, the chance of metastasis being substantial even when the primary tumour has reached a thickness of no more than few millimetres. The metastatic potential of melanocytes has been attributed to the expression of SLUG (SNAIL2), which is absent from HDFs and HMECs, and the silencing of which inhibits metastasis of transformed melanocytes (Gupta et al., 2005). SLUG is a member of the SNAIL super family comprising transcription factors involved in neural crest migration. It is expressed in normal melanocytes and naevi (see next section), suggesting that embryonic differentiation proteins involved in the migratory phenotype of melanocyte precursors predispose oncogenically transformed melanocytes to become highly metastatic.

ACTIVATING BRAF MUTATIONS IN MELANOCYTIC NAEVI AND OTHER BENIGN HUMAN LESIONS

The term 'benign' denotes tumours that show no or very limited propensity to invade surrounding tissues and lack metastatic potential. In general, the initial growth of a benign tumour is followed by stabilization in size and loss of most or all proliferative activity. As a result, benign tumours seldom pose a threat to the patient, unless they happen to be located at a body site where local tumour mass effect compromises a vital body function. Benign tumours can share oncogenic mutations with their malignant counterparts and some are therefore considered to be precursor lesions with the potential to progress to malignancy, probably as a function of additional genetic alterations. A good example is the melanocytic naevus (mole), a small benign tumour of cutaneous melanocytes. Melanocytic naevi, which are exceedingly common, can be regarded as the benign counterparts of melanoma, and occasionally give rise to melanoma. Like melanomas, naevi frequently harbour

activating *BRAF* mutations (82%; Pollock et al., 2003). In combination with the prominent role of $BRAF^{E600}$ in melanoma as described above, this suggests that BRAF is involved already in the early stages of melanoma development, at least in those melanomas that arise within a naevus (Figure 4).

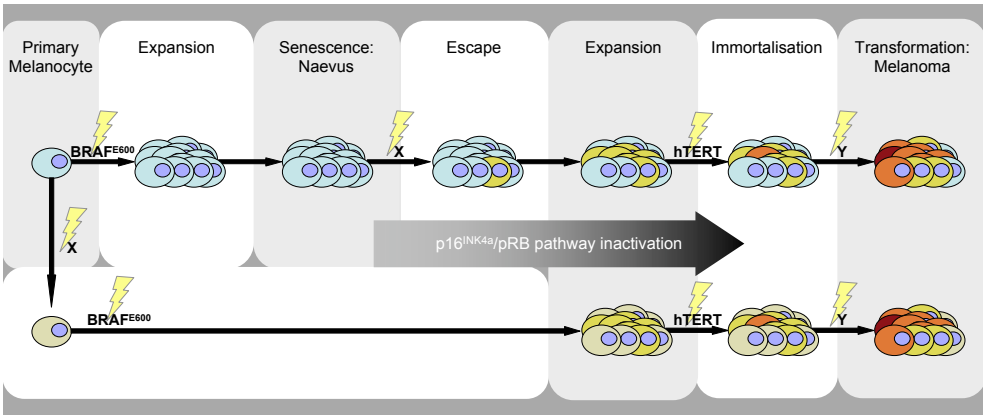

Figure 4 | ***Model of BRAF^{E600}-induced naevus and melanoma formation.*** Melanocytic naevi are thought to originate from the clonal expansion of a single melanocyte acquiring a $BRAF^{E600}$ mutation. After an initial phase of proliferation, the naevus cells enter senescence. Escape from senescence requires an (or more) additional, yet to be identified, hit (denoted 'X'), which might collaborate with loss of $p16^{INK4a}$ activity. It is unclear whether 'X' happens in a fully senescent melanocyte, or in a melanocyte on its way to senescence. It is predicted that such a hit triggers the naevus cell to resume proliferation. To gain immortality, however, the cells have to fully overcome replicative senescence by inactivating the $p16^{INK4a}$/pRB pathway and by maintaining a minimal telomere length, which can be achieved by activation of hTERT. Full oncogenic transformation requires yet (an) additional (epi-)genetic hit(s) (denoted 'Y') causing the establishment of a melanoma. Alternatively, when a melanocyte already suffering from hit 'X' acquires a $BRAF^{E600}$ mutation, it might fail to undergo oncogene-induced senescence. However, to overcome replicative senescence and undergo oncogenic transformation, it too requires additional (epi-)genetic hits.

Several types of naevus are distinguished and are associated with different frequencies of *BRAF* mutation. Although a large proportion of common acquired naevi harbour mutant *BRAF*, hardly any such mutations have been identified in Spitz naevi, which typically harbour *HRAS* mutations and/or amplification (see below; Bastian et al., 2000) similarly, BRAF mutations are absent from so-called blue naevi (Yazdi et al., 2003). Although *BRAF* mutations are frequently present in common acquired naevi, their prevalence in congenital naevi (which are present at birth) is less clear. Several groups have reported *BRAF* mutations in congenital naevi (Davies et al., 2002; Michaloglou et al., 2005; Papp et al., 2005; Yazdi et al., 2003). Others

however, did not detect them in congenital naevi of medium to large size and in giant congenital naevi, some of which harbour *NRAS* mutations (De Raeve et al., 2006; Bauer et al., 2007). A possible explanation for this discrepancy could be the differences in size of the congenital naevi studied. Indeed, according to one study there is a marked difference in *BRAF* mutation frequency between small (<15 mm) and medium sized congenital naevi, with the smaller samples harbouring *BRAF* mutations more often than the larger ones (Ichii-Nakato et al., 2006).

Naevi display many of the characteristics of senescent cells *in vivo*, including near-total lack of proliferation (Kuwata et al., 1993; Bennett, 2003). In addition, many naevus cells express elevated levels of $p16^{INK4a}$ (Wang et al., 1996), show increased SA-β-Gal activity (Michaloglou et al., 2005; Gray-Schopfer et al., 2006), and may contain large multi-nucleated cells (Gray-Schopfer et al., 2006). The absence of telomere attrition in naevus cells (Miracco et al., 2002; Michaloglou et al., 2005), which could trigger replicative senescence, together with the presence of an activated oncogene, imply that naevi undergo oncogene-induced, rather than replicative senescence. As they progress to malignant melanoma only very rarely, naevus-associated oncogene-induced senescence appears to act as a highly reliable *in vivo* fail-safe mechanism protecting from tumourigenesis (Mooi & Peeper, 2006).

Intriguingly, *BRAF* mutations are found also in serrated polyps of the colon, benign epithelial mucosal neoplasms that are the precursors of some colorectal carcinomas. Both serrated polyps and colorectal carcinomas often carry *BRAF* mutations in association with DNA methylation (Domingo et al., 2004) and microsatellite instability (Chan et al., 2003; Kambara et al., 2004; Yang et al., 2004; reviewed in Goldstein, 2006). Benign tumours such as serrated polyps differ from naevi since they are not associated with complete lack of proliferation and, as such, cannot be considered typically senescent (Minoo & Jass, 2006). Although at this point we can only guess which factors determine whether or not benign lesions can undergo proliferative arrest, they might include the microenvironment as well as the cell type. A combination of proliferation and senescence markers can provide the senescence index, reflecting the ratio of proliferating *versus* arrested cells. This could serve as a diagnostic tool, indicating tumour stage or aggressiveness, although it would probably require better-defined senescence markers (Collado & Serrano, 2006). Nevertheless, benign tumours can be precursors of carcinomas and as such represent a setting in which a genuine senescence-associated tumour suppressive mechanism operates in response to oncogenic signalling. Typical senescence pathways, therefore, seem to be involved in limiting the cellular proliferative potential in various settings and to various extents.

Pathways involved in senescence induction in naevi

The presence of increased $p16^{INK4a}$ levels in naevi implies the involvement of

this tumour suppressor in their stable cell cycle arrest. Considering its frequent inactivation in melanoma, $p16^{INK4a}$ is an established melanoma tumour suppressor gene in humans. Germline mutations in *p16^{INK4a}* and *CDK4* ($CDK4^{R24C}$) are associated with familial melanoma (Hussussian et al., 1994; Zuo et al., 1996) and *p16^{INK4a}* knockout mice treated with DMBA develop, amongst other tumours, melanoma (Krimpenfort et al., 2001; Sharpless et al., 2001). Furthermore, in about 80% of human sporadic melanoma cases, $p16^{INK4a}$ function is lost by deletion (Curtin et al., 2005), mutation (COSMIC database; Forbes et al., 2006) or promoter methylation (Straume et al., 2002) (Figure 4). Alternatively, the pRB-$p16^{INK4a}$ pathway is inactivated by mutation, amplification and/or overexpression of genes suppressing pRB activity, like *CDK4* and *CCND1* (Curtin et al., 2005; Errico et al., 2003; Wang et al., 1996). Although $p16^{INK4a}$ is upregulated in human naevi and ectopic overexpression of $p16^{INK4a}$ in cultured cells is sufficient to induce proliferative arrest, individuals homozygous for a $p16^{INK4a}$-inactivating mutation still develop growth-arrested naevi, albeit at increased number and size (Gruis et al., 1995). In addition, naevi of individuals carrying wt *p16^{INK4a}* alleles often display a mosaic pattern of $p16^{INK4a}$ expression even though virtually all the naevus cells are arrested and display increased SA-β-Gal activity (Michaloglou et al., 2005; Gray-Schopfer et al., 2006). It thus appears that $p16^{INK4a}$ collaborates with other, yet to be identified, genes to induce long-term cell cycle arrest in human naevus cells. Loss of $p16^{INK4a}$ function is sufficient to immortalise primary human melanocytes expressing hTERT and endows both primary melanocytes and HDFs with increased proliferative capacities (Gray-Schopfer et al., 2006; Michaloglou et al., 2005; Sviderskaya et al., 2003). It is possible, therefore, that during melanoma development loss of $p16^{INK4a}$ function provides a proliferative advantage rather than circumvents senescence *per se*. This view is further supported by the correlation between loss of $p16^{INK4a}$ expression and increased proliferation in human melanoma (Talve et al., 1997; Straume et al., 2000).

Although a substantial percentage of germline mutations and deletions affecting the *CDKN2A* locus target only *p16^{INK4a}*, several familial melanoma cases carry mutations affecting both *p16^{INK4a}* and *ARF* genes (Ruas & Peters, 1998) or only *ARF* (Rizos et al., 2001; Randerson-Moor et al., 2001). Furthermore, in mouse models, melanocyte-specific overexpression of $HRAS^{V12}$ (Chin et al., 1997; Sharpless et al., 2003) or $NRAS^{K61}$ (Ackermann et al., 2005) cooperates with loss of either $p16^{INK4a}$ or ARF in the induction of melanoma. Interestingly, in either setting a high frequency of somatic inactivation of the adjacent partner gene is observed in the tumours, indicating that $p16^{INK4a}$ and ARF cooperate at least in mice to suppress the development of melanoma *in vivo* (Sharpless et al., 2003). These data suggest that ARF has an independent contribution to melanoma development. As has been noted previously (Bennett & Medrano, 2002), it is remarkable that three melanoma susceptibility

genes (*p16^{INK4a}*, *CDK4* and *ARF*) are key mediators of cellular senescence. p53, which is regulated by ARF, is not frequently inactivated in melanoma (reviewed in Hussein et al., 2003), although it is the most frequently mutated tumour suppressor in human cancer (reviewed in Levine et al., 1991). Furthermore, neither p53 nor p21^{CIP1} is generally induced in naevi (Lassam et al., 1993; McGregor et al., 1993; Maelandsmo et al., 1996; Trotter et al., 1997; Gray-Schopfer et al., 2006). So, it seems that the role of p53 in melanoma progression is less prominent than in other tumour types. Loss of ARF however, could act as a p53 pathway-inactivating lesion. Indeed, other upstream and downstream targets of p53 have not been studied extensively in melanoma, leaving the possibility that the p53 pathway is subject to inactivation at different levels. Another tumour suppressor implicated in melanoma development is the phosphatase and tensin homologue PTEN, which is found mutated in approximately 17% of human melanomas (Guldberg et al., 1997; COSMIC database; Forbes et al., 2006). An alternative mechanism for PTEN inactivation is epigenetic silencing of its promoter, which is observed in approximately 25% of primary and up to 60% of metastatic melanoma (Mirmohammadsadegh et al., 2006). Interestingly, *PTEN* mutations are often found together with *BRAF* mutations, whereas they are mutually exclusive with *RAS* mutations (Daniotti et al., 2004; Tsao et al., 2004). It is still unclear however, if PTEN has a role in naevus formation as well as whether loss of PTEN function contributes to early or/and late melanoma development. Finally, UPR is another mechanism suggested to be involved in the proliferative arrest of Spitz naevi, which display enhanced staining for the ER-associated unfolded protein response sensor Grp78 (see above; Denoyelle et al., 2006).

Recent data reveal that cells undergoing oncogene-induced senescence *in vitro* display signs of a DNA damage response (DDR), which is causally involved in senescence induction (Bartkova et al., 2006; Di Micco et al., 2006; Mallette et al., 2007). Such DDR has been demonstrated also in premalignant lesions *in vivo*, which often harbour a significant pool of proliferating cells (Bartkova et al., 2005; Gorgoulis et al., 2005; Bartkova et al., 2006; Di Micco et al., 2006). Considering proliferative arrest as a central theme in senescence, analysis of the proliferative activity of benign tumours at the single-cell level will facilitate assessing the contribution of the DDR pathways to the onset of senescence *in vivo*.

ERK activity in benign lesions

As discussed in a previous section, BRAF-activating mutations result in increased p-ERK levels in vitro, predicting that this may happen also in BRAF^{E600}-expressing tumours. The presence of p-ERK, however, appears to correlate with proliferative activity in the tumour rather than with the presence of a *BRAF* mutation, as only 23% of common naevi with BRAF^{E600} show p-ERK positivity (Uribe et al., 2006). In agreement with this, only 7.1% of Papillary Thyroid Carcinoma (PTC) with BRAF^{E600},

tumours with a low proliferative index, show detectable p-ERK levels in >1% of the tumour cells (Zuo et al., 2007). A note of caution is that immunohistochemical detection of p-ERK does not always correlate with Western Blot detection of pERK (Takata et al., 2005). Furthermore, ERK is not a direct target of BRAF (Figure 2) and therefore ERK phosphorylation -mediated by active MEK- is an indirect measure of BRAF activity, subject also to BRAF-independent regulation. Therefore, MEK phosphorylation status might represent a better indication of BRAF activity. Nonetheless, there seems to be a discrepancy between the large body of *in vitro* data and *in situ* analyses. Such an inconsistency could be the result of the fact that *in vitro* experiments often involve overexpression of mutant BRAF, which is not (yet) seen in naevi and PTCs. In contrast to PTC, normal thyroid follicular cells do display high levels of p-ERK (Zuo et al., 2007), which suggests that the RAS-MEK-ERK pathway is fully functional in these cells. It is therefore unlikely that the acquisition of a BRAF-activating mutation is not sufficient to induce p-ERK in these lesions. Perhaps in lesions such as naevi and PTC, ERK activity is maintained at a constitutive but low level that is difficult to detect. Indeed, it will be important to determine whether naevi are completely devoid of $BRAF^{E600}$-ERK signalling. Another possibility is that constitutive BRAF signalling is dampened *in vivo* by way of negative feedback loops, perhaps including BRAF inhibitors and/or ERK phosphatases (Schreck & Rapp, 2006; Kolch, 2005). Such a negative feedback loop has recently been described for RAS in neurofibromas (Courtois-Cox et al., 2006) and if extrapolated to BRAF, could explain the lack of ERK activation in indolent $BRAF^{E600}$ tumours. In this regard, one might hypothesize that subsequent loss of a critical component of such a feedback loop would promote tumorigenesis. Alternatively, $BRAF^{E600}$-induced ERK phosphorylation could be a short-lived phenomenon that has ceased by the time the lesions are analysed. In the case of naevi, for example, transient $BRAF^{E600}$-mediated ERK activation could contribute to the formation of the lesion from a single melanocyte. Once senescence is established in naevus cells, ERK activity might be suppressed. Consistent with this idea, CRAF-induced senescence *in vitro* has been demonstrated to be irreversible, as it is maintained upon downregulation of activated CRAF (Zhu et al., 1998).

$BRAF^{E600}$ MODEL ORGANISMS

The first model organism used to study the role of $BRAF^{E600}$ *in vivo* was the zebrafish (Patton et al., 2005). Like other vertebrates including man, these fish have neural crest-derived melanocytes that express, and are dependent on, MITF. Transgenic zebrafish expressing wt BRAF under control of the melanocyte-specific *mitfa* promoter fail to show any phenotype. In contrast, by 8 weeks of development, 10% of $BRAF^{E600}$-transgenic fish develop focal sites of melanocyte proliferation,

which manifest as well-differentiated, stable lesions. These 'fish-naevi' resemble their human counterparts and fail to progress to malignancy. However, in the context of p53 inactivation, $BRAF^{E600}$ fish develop malignant melanomas, whose histology resembles human melanoma. The melanomas are transplantable, display genomic instability and show p-ERK staining. In addition to serving as a model for melanomagenesis, these fish can be used for screens for melanoma-suppressing or -enhancing genes as well as for testing novel melanoma therapeutics (Patton et al., 2005; Stern & Zon, 2003).

Several $BRAF^{E600}$ mouse models have been generated also. A transgenic mouse model targeting $BRAF^{E600}$ expression specifically to thyroid cells shows increased p-ERK staining and an enlarged thyroid. It develops PTC with tall cell features and closely resembles human PTC with $BRAF^{E600}$. Tumours of the transgenic line with high expression of $BRAF^{E600}$ display areas of invasion and foci of poorly differentiated carcinoma, whereas the transgenic line with low $BRAF^{E600}$ expression develops smaller tumour foci with lower penetrance and a less aggressive phenotype (Knauf et al., 2005).

Two Cre-recombinase (Cre)-mediated $BRAF^{E600}$ knock-in mouse models have recently been generated. These are highly useful models to investigate the effects of inducible and tissue-specific expression of endogenous $BRAF^{E600}$ at physiological levels. In the first model, Cre-mediated $BRAF^{E600}$ expression during development results in embryonic lethality (Mercer et al., 2005). When interferon-inducible Cre is used, $BRAF^{E600}$ is expressed in many tissues, but only liver and spleen display moderately increased proliferation. Furthermore, the mice develop non-lymphoid neoplasia of the histiocytic type, but die within 4 weeks due to bone marrow failure (Mercer et al., 2005). In the second inducible knockin model, in which expression is confined to the lung epithelium, $BRAF^{E600}$ results in the development of multiple lung adenomas (Dankort et al., 2007). These lesions appear to be dependent on MEK/ERK signalling, as is indicated by the prevention of adenoma development upon pharmacological inhibition of MEK. Importantly, when untreated, the adenomas grow rapidly until ~8 weeks, but subsequently display a dramatic reduction in proliferative activity. This is accompanied by the induction of the senescence-associated protein ARF in a subset of the cells, as well as by a newly discovered senescence marker, DEC-1 (Collado et al., 2005). This is similar to what has been observed for a $KRAS^{V12}$ knockin lung adenoma model, although those adenomas display also induction of additional senescence markers (SA-β-Gal, $p16^{INK4a}$, $p15^{INK4b}$ and DcR2; Collado et al., 2005). The $BRAF^{E600}$-induced adenomas rarely progress to adenocarcinomas, unless either *p53* or *CDKN2A* is deleted (Dankort et al., 2007). Although results from mouse models may not necessarily be directly extrapolated to the human situation, as for example the localisation of melanocytes in mouse skin

is different from that in human skin, it will still be very informative to determine the effect of $BRAF^{E600}$ in melanocytes or thyroid cells, also in the context of other cancer-predisposing lesions.

$BRAF^{E600}$ AS A THERAPEUTIC TARGET

Inhibition of MEK by the synthetic compounds U0126 or CI 1040 in mutant BRAF-expressing cell lines reduces p-ERK, inhibits proliferation and oncogenic transformation (Calipel et al., 2003; Solit et al., 2006). In some cell lines it induces apoptosis, which is accompanied by a decline in cyclin D1 levels and an upregulation of the CDK inhibitor $p27^{KIP}$ (Calipel et al., 2003). Most BRAF and some RAS mutant tumour cell lines have been reported to be highly sensitive to CI 1040, whereas cell lines without *BRAF* or *RAS* mutation are generally resistant (Solit et al., 2006), although it is not entirely clear whether the melanocytic origin contributes to this resistance. However, all cell lines that carry a mutant *BRAF* allele, independent of their origin, are highly sensitive to CI 1040, which is reinforced by the finding that a sensitive breast cancer cell line with previously unknown *BRAF* mutational status appeared to harbour a BRAF mutation (Solit et al., 2006). Consistent with MEK inhibition *in vitro*, PD0325901, a CI 1040 derivate, suppresses the growth of s.c. xenografts of mutant BRAF melanoma cell lines, correlating with reduced levels of the D-type cyclins and p-pRB and an induction of $p27^{KIP}$ (Solit et al., 2006). Furthermore, CI 1040 inhibits tumour invasion and metastasis of mutant BRAF-expressing cell lines injected intravenously (i.v.) into immunodeficient mice (Collisson et al., 2003). This could be explained by the finding that $BRAF^{E600}$ signals via MEK/ERK to induce MMP-1, which results in collagen digestion and the ability to invade matrigel (Huntington et al., 2004). Also β3- and α6-integrin may be involved; they are important in melanoma invasion and metastasis and are downregulated by MEK inhibition in mutant BRAF melanoma cell lines (Woods et al., 2001). These data show that $BRAF^{E600}$-associated tumours are highly dependent on MEK, which, therefore, constitutes a promising target for cancer therapy. Several MEK inhibitors are currently tested in clinical trials in melanoma patients (reviewed in Gray-Schopfer et al., 2007). Results from *in vitro* and *in vivo* experiments with BAY43-9006 (also known as Sorafenib and Nexavar), an inhibitor of RAF kinases, VEGFR and PDGFR (Wilhelm et al., 2004), are similar those obtained with MEK inhibitors in melanoma and thyroid cell lines (Karasarides et al., 2004; Sharma et al., 2005; Salvatore et al., 2006). Treatment of s.c. xenografts of $BRAF^{E600}$-expressing melanoma cell lines with BAY43-9006 inhibits MEK activation and reduces tumour volume, although the tumours eventually relapse (Karasarides et al., 2004). Tumours from mice treated with BAY43-9006 display less vascularisation, which is followed by a reduction in the number of cycling cells and increased apoptosis (Sharma et al., 2005). Furthermore, treatment

with BAY43-9006 results in reduced VEGF secretion *in vitro*, while si-RNAs targeting VEGF expression inhibit tumour growth *in vivo*. This is in contrast with previous data showing that sh-BRAF does not have an effect on tumour vascularisation (Hoeflich et al., 2006), which might be explained by the use of different melanoma cells and the fact that BAY43-9006, as opposed to the BRAF sh-RNA, does not target BRAF exclusively. Subsequent studies on the same cells injected i.v. to investigate its effect on experimental lung metastasis show that the MEK inhibitor, but not the BRAF inhibitor, reduces metastasis (Sharma et al., 2005). In agreement with this observation, comparison of CI 1040 to BAY43-9006 in a ΔCRAF transgenic lung adenoma mouse model demonstrates that, although the two inhibitors behave similarly in an *in vitro* kinase assay and in cell lines, only CI 1040 reduces tumour formation (Kramer et al., 2004). Possibly, BAY43-9006 does not always reach the tumour cells sufficiently, or targets mainly VEGFR *in vivo*. Overall, these experiments show that in melanoma and other cancers, BRAF constitutes a therapeutic target that can be inhibited either directly or at a downstream level. Although BAY43-9006 has not shown the marked anti-melanoma activity in the clinic as it did in some mouse models, other BRAF inhibitors that are currently being generated and tested might prove more potent (reviewed in Gray-Schopfer et al., 2007).

It might be worth considering developing inhibitors that target multiple RAF family members simultaneously. An sh-RNA specifically targeting $BRAF^{E600}$ induces apoptosis in homozygous mutant *BRAF* melanoma cell lines, but the existence of one wt *BRAF* allele is sufficient to block these effects in the presence of growth factors (Christensen & Guldberg, 2005). Since similar factors are likely to be present in the microenvironment, they may still activate the RAS/MEK/ERK pathway in melanocytes through wt BRAF, thereby interfering with the efficacy of any drug specifically targeting $BRAF^{E600}$. Indeed, inhibition of both wt and mutant BRAF in heterozygous cell lines results in apoptosis even in the presence of growth factors, stressing the need for a BRAF inhibitor targeting both wt and mutant BRAF (Christensen & Guldberg, 2005). Whether ARAF or CRAF can functionally compensate for BRAF is depending on the cell line. The downregulation of CRAF does not affect the *in vitro* and *in vivo* proliferative characteristics of certain melanoma cell lines (Sharma et al., 2005; Hingorani et al., 2003) and in those cell lines that are sensitive, it does not do so via the MEK/ERK pathway (Karasarides et al., 2004). Furthermore, inhibiting the entire RAF family would impact also on tumours with *RAS* mutations (CRAF being the preferred RAS effector; Dumaz et al., 2006) and tumours with CRAF overexpression (Figure 3). In fact, U0126 treatment or knockdown of CRAF, but not ARAF or BRAF, inhibits p-ERK, cell proliferation and induces apoptosis in ovarian cancer cell lines overexpressing CRAF (McPhillips et al., 2006). Although most experiments have been performed *in vitro*, the results suggest that

an inhibitor targeting both $BRAF^{E600}$ and wt BRAF, as well as ARAF and CRAF might be more effective and applicable to a broader range of tumours.

Combining BRAF and/or MEK inhibitors with other drugs might increase the therapeutic window (Gray-Schopfer et al., 2007). For example, targeting the PI3K pathway, which is likely to interfere with mutant RAS-driven tumourigenesis, might also impact on mutant BRAF tumours. There are several ways to activate the PI3K pathway, of which PTEN inactivation (Guldberg et al., 1997; Mirmohammadsadegh et al., 2006) and overexpression of AKT3 (Stahl et al., 2004) are most frequent in melanoma. Melanoma cell lines exposed to silencing of $BRAF^{E600}$ cannot survive independently of anchorage, but can do so in the presence of a fibronectin matrix. The latter is accompanied by induction of p-AKT, and survival can be inhibited by treatment with the PI3K inhibitor LY294002 (Boisvert-Adamo & Aplin, 2006). Hence interference with both MEK and PI3K signalling may be more effective against melanoma, also *in vivo*. Simultaneous inhibition of the RAS/RAF/MEK and PI3K pathways has been shown to induce apoptosis in melanoma cell lines more efficiently than each alone (Krasilnikov et al., 2003; Molhoek et al., 2005; Meier et al., 2007). Furthermore, several receptor tyrosine kinases, such as c-MET, type I insulin-like growth factor receptor (IGFR) and cKIT, which activate RAS, are overexpressed in malignant melanoma (reviewed in Meier et al., 2005). Downregulation of the IGFR in some mutant BRAF melanoma cell lines inhibits survival, which is probably due to loss of PI3K signalling (Yeh et al., 2006; Calipel et al., 2003).

Another approach to target tumours harbouring mutant *BRAF* is to take advantage of the increased dependency of this mutant protein for the heat shock protein 90 (HSP90), a chaperone that facilitates proper protein folding (da Rocha Dias et al., 2005; Grbovic et al., 2006). HSP90 has emerged to be of great importance to cancer cells. It is expressed at elevated levels in tumor cells and contributes to their proliferative capacity and survival. Other HSP90 clients include oncoproteins such as CRAF, CDK4, AKT, mutant p53 and hTERT (reviewed in Isaacs et al., 2003; Sharp et al., 2006). Preclinical data from patients with melanoma or other tumours indicate that heat-shock protein HSP90 inhibitors (*e.g.* geldanamycin or 17AAG) display anticancer activity, making them promising drugs for melanoma treatment (reviewed in Sharp et al., 2006).

FUTURE CHALLENGES

Since the discovery of *BRAF* mutations in human cancer in 2002, its role in tumourigenesis has attracted widespread interest. Indeed, several important questions have been addressed. For example, we now know that in many tumour cell lines persistent $BRAF^{E600}$ expression is required to maintain their proliferative, survival and oncogenic potential. Furthermore, with the use of diverse animal models that

recapitulate the pathophysiologic situation in man, we have learned that $BRAF^{E600}$ contributes to the formation of benign lesions, which progress to malignancy only in the context of specific additional genetic mutations.

Several fundamental questions have, however, remained unanswered. BRAF is a member of a signalling cascade that is fine-tuned by a variety of positive and negative regulators. Well-known members of the RAS/MEK/ERK pathway, such as RAS and BRAF, are frequently mutated in a variety of cancers. Although appealing hypotheses have been put forth, it is still unclear why *BRAF* mutations are selected for preferentially in melanoma and thyroid cancer. It will be interesting to know whether these two cell types are more likely to acquire *BRAF* mutations, or perhaps share certain signals that collaborate in oncogenic transformation with mutations in *BRAF*. If the latter were true, one would expect a BRAF signature to be deduced from expression microarray data. Although this has been established for thyroid tumours, no such characteristic has been made for melanoma, which could reflect technical problems such as the use of melanoma cell lines rather than tumour specimens, or the common presence of 'contaminating' nonneoplastic cells.

Another question relates to the molecular basis underlying the high frequency of *BRAF* mutations in cutaneous melanoma. Although melanoma has been strongly linked to UV exposure, the mechanism of acquisition of the $BRAF^{T1799A}$ mutation remains to be clarified. The existence of this *BRAF* mutation also in tumours sheltered from UV exposure indicates the presence of alternative mechanisms. The identification of this putative mutation-inducing mechanism will likely provide molecular insight into the events triggering the early steps of tumourigenesis.

Regarding oncogene-induced senescence *in vivo*, various challenges remain. For example, there is a strong need to expand the number of genes that are causally involved in the cessation of proliferation. Recently reported factors like Dec1 and DcR2 are interesting new candidates (Collado et al., 2005). Proteins like these and others, yet to be identified, may serve not only as novel diagnostic tools, but will conceivably elucidate the signalling network implementing senescence *in vivo*. Along these lines, it will be important to uncover the fundamental parameters determining different outcomes of the activation of oncogenic signalling pathways *in vivo*. Indeed, oncogenic mutations may fail to induce any biological effect at the one-cell stage, may result in either apoptosis or senescence at a time that the lesion is still very small, may induce initial expansion followed by induction of senescence, or even straight progression to malignancy (Mooi & Peeper, 2006). It is this type of information that will allow us to estimate the true dimensions and effectiveness of senescence induced by oncoproteins like $BRAF^{E600}$.

We and others have shown previously that naevi, which often harbour the $BRAF^{E600}$ mutation, display hallmarks of senescence. Serrated polyps of the colon constitute

another benign lesion, which often harbour the same mutation and have been proposed to represent another example of a benign tumour associated with oncogene-induced senescence *in vivo* (Minoo & Jass, 2006). Are there any more benign lesions expressing the oncogenic $BRAF^{E600}$ protein? Interestingly, *BRAF* mutations are found in benign ovarian cystadenomas, although it is not yet clear whether these lesions display senescence characteristics (Ho et al., 2004). *BRAF* mutations have also been reported for papillary microcarcinoma (PMC) of the thyroid. Although this tumour is generally not considered benign, it can remain asymptomatic throughout life. Further characterisation of these lesions may reveal a benign subset that is associated with senescence (Nikiforova et al., 2003; reviewed in Baloch & LiVolsi, 2006).

Finally, the high prevalence of *BRAF* mutations in human tumours, as well as the fact that the BRAF kinase and some of its downstream effectors represent pharmacologically tractable targets, have fuelled the rapid development of clinically exploitable inhibitors. These drugs have yielded encouraging results *in vitro* and are being tested in clinical trials (reviewed in Gray-Schopfer et al., 2007). With the incidence of melanoma rising, development of drugs targeting BRAF, the entire RAF family, or its effectors, like MEK should present us with strategies for improved and customised therapeutic intervention of $BRAF^{E600}$-harbouring human tumours. Resolving the mechanism leading to the specific $BRAF^{E600}$ mutation, as well as increasing our understanding of the signalling network downstream of $BRAF^{E600}$ might yield additional therapeutic opportunities.

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$BRAF^{E600}$-associated senescence-like cell cycle arrest of human nevi

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BRAF^{E600}-associated senescence-like cell cycle arrest of human nevi

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ABSTRACT

Most normal mammalian cells have a finite lifespan (Hayflick, 1965), thought to constitute a protective mechanism against unlimited proliferation (Mathon and Lloyd, 2001; Lowe *et al.*, 2004; Campisi, 2005). This phenomenon ('senescence') is driven by telomere attrition, which triggers the induction of tumor suppressors, including p16^{INK4a} (Shay and Roninson, 2004). In cultured cells, senescence can be elicited also prematurely, by oncogenes (Serrano *et al.*, 1997). However, whether such Oncogene-Induced Senescence (OIS) represents a physiological process has long been debated. Human nevi ('moles'), benign tumours of melanocytes, frequently harbour oncogenic mutations in BRAF (Pollock *et al.*, 2003) (predominantly V600E). Nonetheless, nevi typically remain growth-arrested for decades and only rarely progress into malignancy (melanoma) (Kuwata *et al.*, 1993; Bennett, 2003; Chin *et al.*, 1998). This raises the question whether nevi undergo BRAF^{V600E}-induced senescence. We report here that sustained BRAF^{V600E} expression in human melanocytes induces cell cycle arrest, which is accompanied by the induction of p16^{INK4a} as well as of a commonly used senescence marker, Senescence-Associated acidic β-Galactosidase (SA-β-Gal) activity. Validating these results *in vivo*, congenital nevi are invariably SA-β-Gal-positive, showing for the first time this classical senescence-associated marker in a -largely growth-arrested-

neoplastic human lesion. In growth-arrested melanocytes, both *in vitro* and *in situ*, we observe a striking mosaic induction of $p16^{INK4a}$, suggesting that factors other than $p16^{INK4a}$ also contribute to protection against $BRAF^{E600}$-driven proliferation. Finally, nevi do not appear to suffer from telomere attrition, arguing in favour of an active oncogene-driven senescent process, rather than a loss of replicative potential. Thus, both *in vitro* and *in vivo*, $BRAF^{V600E}$-expressing melanocytes display classical hallmarks of senescence, suggesting that OIS represents a genuine protective physiological process.

RESULTS AND DISCUSSION

Melanocytic nevi represent an intriguing human setting where an activated oncogene can co-exist with long-term arrested cells. Nevi are very common, clonal and benign tumours of cutaneous melanocytes (Robinson *et al*., 1998). They frequently harbour the V600E mutation in BRAF (Pollock *et al*., 2003), a protein kinase and downstream effector of RAS (NCBI gene bank re-named the V599E mutation based on newly available sequence data; accession number: NM_004333.2; hereinafter referred to as $BRAF^{E600}$). Yet, in spite of the oncogenic nature of this mutation (Davies *et al*., 2002; Wellbrock *et al*., 2004), an initial phase of nevus growth is typically followed by a near-complete cessation of proliferative activity, which is maintained for many decades (Kuwata *et al*., 1993; Bennett, 2003; Mooi and Krausz, 1992). Therefore, it is conceivable that growth arrest of nevi results from OIS, acting as an effective cellular brake against $BRAF^{E600}$-mediated oncogenic signaling.

To test this hypothesis, we first determined the effect of the nevus-associated $BRAF^{E600}$ mutant on the proliferative capacity of freshly isolated normal human skin melanocytes. A bicistronic lentiviral vector, co-expressing $BRAF^{E600}$ and eGFP (to monitor infection efficiency, typically approximately 90%; Figure 1a) was used to transduce melanocytes. Short-term expression of $BRAF^{E600}$ (3-7 days) led to enhanced melanocyte proliferation, as measured by a moderate but reproducible increase in BrdU incorporation (Figure 1b and data not shown). However, this effect was transient: sustained expression of $BRAF^{E600}$ resulted in a marked cell cycle arrest. In the vast majority (roughly 80%) of transduced melanocytes, this was associated with an intense activity of Senescence-Associated β-Galactosidase (SA-β-Gal), a widely used marker for senescent or stressed cultured cells as well as for aged tissues *in vivo* (Dimri *et al*., 1995; Serrano *et al*., 1997) (Figure 1c).

The $p16^{INK4a}$ protein is a major tumour suppressor, often highly expressed in senescent cells *in vitro* and inactivated in a variety of human cancers, including 30-70% of melanomas (Sharpless and Chin, 2003). We found that $BRAF^{E600}$-expressing melanocytes had elevated levels of $p16^{INK4a}$ (Figure 1d). Remarkably, the staining for $p16^{INK4a}$ was heterogeneous, with 25-35% of the $BRAF^{E600}$-expressing melanocytes

showing low or undetectable p16^{INK4a} levels, in spite of the fact that over 95% of these cells were arrested (and still expressed the lentiviral cassette, as evident from eGFP-positivity).

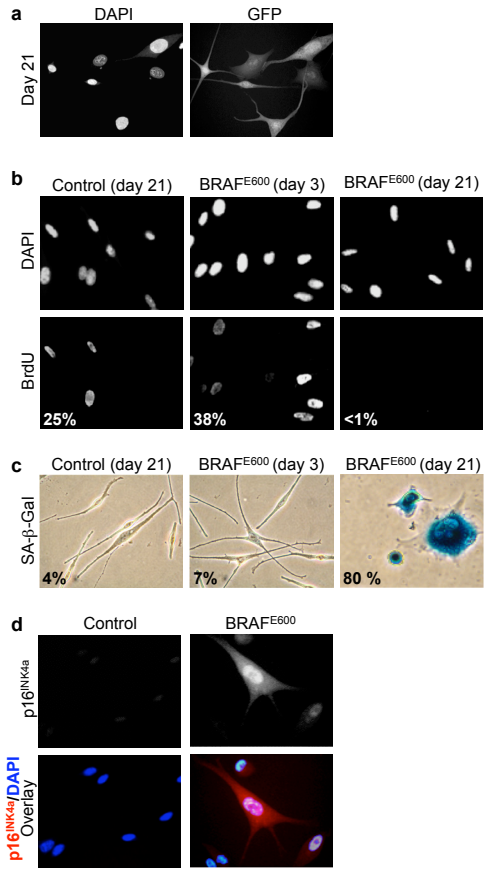

Figure 1 | ***Sustained expression of BRAF^{E600} induces a senescence-like arrest of normal human melanocytes.*** **a |** Infection efficiency estimated to be approximately 90%, by visualization of eGFP-positive cells. Nuclear staining by DAPI (in blue) is shown as an indication of total cell number. **b |** Impact of BRAF^{E600} on melanocyte proliferation measured as the percentage of positive cells after a 3 h-pulse with BrdU. (Top) DAPI stain to detect nuclei. (Bottom) BrdU-positive cells. Control melanocytes remained proliferative and viable. **c |** SA-β-gal activity after brief and sustained BRAF^{E600} expression. **d |** Heterogeneous levels of p16^{INK4a} (red fluorescence) in BRAF^{E600}-transduced melanocytes. DAPI stain is used to detect nuclei. Note that, at 21 days post-infection, <1% of the BRAF^{E600}-expressing melanocytes incorporated BrdU, the vast majority showed positive SA-β-gal activity, whereas a smaller proportion of BRAF^{E600}-expressing cells had detectable p16^{INK4a} expression. Numbers given are representative of three independent experiments.

As OIS *in vitro* has been defined best in primary diploid fibroblasts, we analyzed the cellular response to activated BRAF also in this 'reference' context. Consistent with previous results obtained with overexpression of RAS^{V12} or Raf1 (Serrano *et al.*, 1997; Zhu *et al.*, 1998; Lin *et al.*, 1998), retroviral delivery of BRAF^{E600} into two different strains of normal human fibroblasts caused a complete cessation of proliferation, along with induction of p16^{INK4a} (Supplementary Figure 1a and b). To exclude the possibility that these effects were caused by supra-physiologic levels of BRAF^{E600} expression, we designed a system in which its expression levels could be manipulated with considerable precision. Cells were transduced with a mixture of retrovirus encoding a BRAF^{E600} expression cassette, and retrovirus encoding a short hairpin (sh) RNA targeting both endogenous wild type and ectopic BRAF^{E600} (for

details, refer to legends to Figure 2). This strategy allowed expression of BRAF^{E600} to levels similar to those seen in melanoma cells (Supplementary Figure 2a) or even to levels close to, or indistinguishable from, endogenous BRAF levels (Figure 2a, compare lanes 4-5).

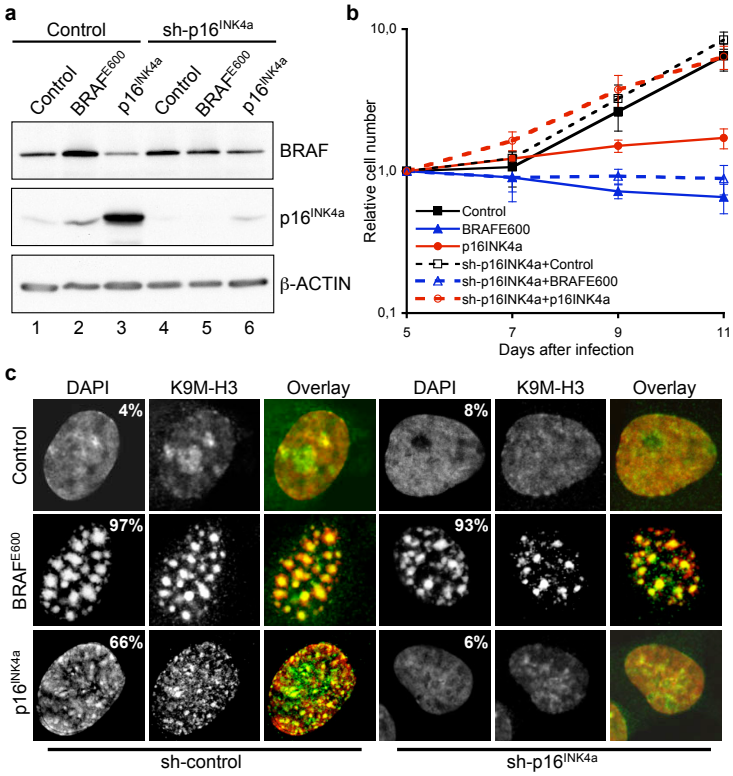

Figure 2 | ***Physiologic levels of BRAF^{E600} induce senescence-like arrest of normal human fibroblasts in a p16^{INK4A}-independent manner.*** **a-c |** Human BJ$_{hTERT}$ cells stably expressing either control or p16^{INK4a} shRNA were transduced with a mixture of a retrovirus encoding BRAF^{E600} expression and puromycin resistance cassettes, and a retrovirus encoding a shRNA targeting both endogenous wild type and ectopic BRAF^{E600} and blasticydin cassettes. Pharmacologic selection for both resistance markers was used to create cells that had stably integrated both the BRAF^{E600} expression cassette and the shRNA. The latter alone did not affect proliferative potential (not shown). As a control, cells were transduced with p16^{INK4a}-encoding retrovirus. **a |** Analysis by Western blotting (β-actin serves as a loading control). **b |** Analysis by proliferation curves (result is from three independent experiments performed in duplicate, shown with standard deviations). **c |** DAPI staining to detect SAHF (percentage of SAHF-positive cells is indicated in insert) and immunofluorescence for K9M-H3. Note that, whereas p16^{INK4a} shRNA neutralized senescence induced by ectopic expression of p16^{INK4a}, it failed to bypass BRAF^{E600}-induced senescence. Even undetectable levels of BRAF^{E600} (**a**, compare lane 4-5) were sufficient to induce growth arrest and SAHF, in the absence of p16^{INK4a}.

The elevation of $p16^{INK4a}$ levels was maintained upon progressively diminishing $BRAF^{E600}$ levels (Supplementary Figure 2a). We did not see upregulation of *p14ARF* by $BRAF^{E600}$ (Supplementary Figure 3). Furthermore, low levels of $BRAF^{E600}$ caused inhibition of both DNA replication (Supplementary Figure 2b) and cellular proliferation (Figure 2b). This arrest was stably maintained, without any significant escape (Supplementary Figure 2c). However, it was bypassed by co-expression of the SV40 large tumour (LT) antigen (Supplementary Figure 2d), arguing against an aphysiologic effect and suggesting that this arrest depends on cellular tumour suppressors. Furthermore, in the context of fibroblasts defective for p53 and $p16^{INK4a}$ and expressing SV40 small tumour (st) antigen and hTERT, $BRAF^{E600}$ could efficiently substitute for RAS^{V12} in the induction of tumours in immunocompromised mice (Supplementary Table 1).

Recently, overexpression of RAS^{V12} has been shown to cause accumulation of senescence-associated heterochromatic foci (SAHF), concentrated spots of transcriptionally silenced DNA (Narita *et al*., 2003). We observed that low levels of $BRAF^{E600}$, too, induced SAHF (Figure 2c). This was accompanied by focal accumulation of a specific heterochromatin-associated histone modification, namely methylation of lysine 9 of histone H3 (K9M-H3). Together, these results indicate that low levels of $BRAF^{E600}$ induce senescence-like cell cycle arrest in primary human cells.

To investigate whether $p16^{INK4a}$ upregulation constitutes a protective response to inappropriate mitogenic signaling by $BRAF^{E600}$, we created cell lines stably expressing $p16^{INK4a}$ short hairpin interfering RNA (shRNA). This shRNA was effective, as it suppressed the accumulation of $p16^{INK4a}$ in various settings (Figure 2a), caused increased proliferation (Supplementary Figure 4) and abolished the induction of cell cycle arrest by overexpressed $p16^{INK4a}$ (Figure 2b; Supplementary Figure 2b and c). However, low levels of $BRAF^{E600}$ inhibited proliferation and induced SAHF even upon $p16^{INK4a}$ depletion (Figure 2b, c; Supplementary Figure 2b and c). Identical observations were made for a $p16^{INK4a}$-deficient binding $CDK4^{R24C}$ mutant (Supplementary Figure 2e), for a different fibroblast strain (TIG3; Supplementary Figure 2f-h) and for fibroblasts explanted from a homozygous $p16^{INK4a}$-deficient 'Leiden' patient (Gruis *et al*., 1995) (Supplementary Figure 2i-l). These results suggest that, at least in cultured human fibroblasts, $p16^{INK4a}$ is upregulated in response to physiologic levels of $BRAF^{E600}$, but is not strictly required for the induction of cell cycle arrest.

Next, we wished to validate *in vivo* our observation that $BRAF^{E600}$ induces senescence-like arrest *in vitro*. Given the high frequency of BRAF mutations in both nevi and melanomas (Pollock *et al*., 2003; Davies *et al*., 2002), we looked for hallmarks of senescence in a panel of resection specimens of human nevi. We first confirmed the presence of the $BRAF^{E600}$ mutation in 8 specimens of our panel of 23 nevi

(Supplementary Figure 5). We then used paraffin-embedded as well as cryosections of normal human skin and resection specimens of nevi for further analysis. Virtually all melanocytes within these nevi were growth-arrested, as judged by negative immunohistochemical staining for the proliferation marker Ki-67 (Figure 3a), in agreement with the literature (Kuwata *et al.*, 1993; Bennett, 2003). This was in contrast to epidermal keratinocytes in the same specimens, many of which showed proliferative activity.

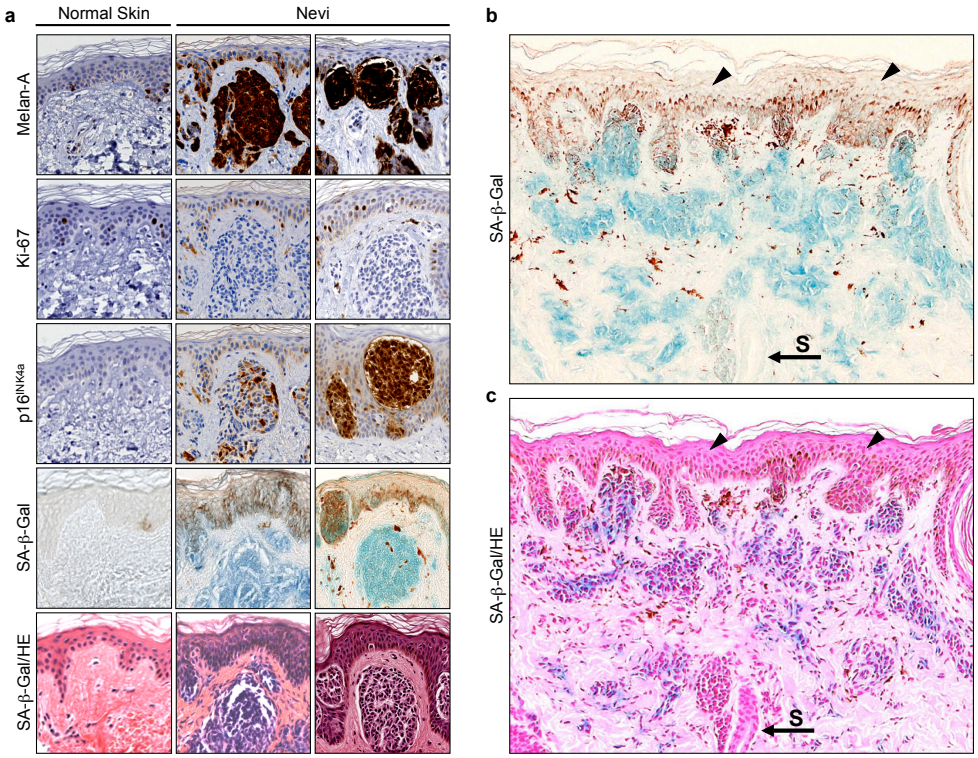

Figure 3 | ***Human melanocytic nevi display hallmarks of senescent cells.*** Paraffin-embedded sections of human nevi and normal skin were subjected to immunohistochemistry with the indicated antibodies. **a |** Melan A (brown) identifies melanocytes, MIB1 (brown) recognizes the proliferation marker Ki-67 and $p16^{INK4a}$ antibody (brown) detects $p16^{INK4a}$. **a**-**c |** Frozen sections of human nevi were subjected to SA-β-Gal staining. The blue staining corresponds exactly to the sites of nevus cell nests. **b** and **c |** All cells of the epidermis (mostly keratinocytes; arrowheads) and sweat gland ('S'), as well as dermal cells between the nevus cell nests are completely negative for SA-β-Gal. Note that the brown staining in these samples comes from pigment, released by the nevus cells. Where indicated, hematoxylin/eosin (HE) was used as counter stain on the consecutive (SA-β-Gal-stained) section to visualize the lesional melanocytes, within the context of the surrounding tissue.

As we had observed that expression of $BRAF^{E600}$ in cultured human melanocytes leads to cell cycle arrest along with induction of SA-β-Gal activity, we subjected our nevi panel to analysis for this commonly used senescence marker. SA-β-Gal activity has been shown previously to increase in human epidermis as a function of age (Dimri *et al.*, 1995). To avoid detection of age-induced SA-β-Gal activity, we analyzed a panel of congenital nevi, obtained from patients under one year of age. Indeed, all 23 nevi analyzed displayed high levels of SA-β-Gal activity (Figure 3a-c). This activity was absent from normal skin melanocytes within the same samples (Figure 3b and c) and in control samples taken from normal skin (Figure 3a). In agreement with previous data (Bandyopadhyay *et al.*, 2001), SA-β-Gal activity was also absent from freshly isolated, cultured primary human melanocytes (Figure 1c). Terminally differentiated keratinocytes within the epidermis of the same samples also did not show SA-β-Gal activity (Figure 3b and c, arrowheads), consistent with previous observations made in young (<39 years of age) donors (Dimri *et al.*, 1995). SA-β-Gal activity was similarly lacking from the cells of skin adnexae, such as the sweat gland (Figure 3b and c, 'S') and from the dermal mesenchymal cells between the nevus cell nests. Thus, human nevi, largely growth-arrested neoplastic lesions, are positive for the common senescence marker SA-β-Gal.

Studies in mouse models and humans indicate that (epi-) genetic inactivation of the *p16INK4a* gene is associated with melanomagenesis (Wang *et al.*, 1996; Bennett, 2003; Sharpless and Chin, 2003; Kamb *et al.*, 1994). Normal melanocytes, scattered alongside the dermal-epidermal junction and surrounded by keratinocytes, had undetectable levels of $p16^{INK4a}$ (Figure 3a). In contrast, nevi invariably contained $p16^{INK4a}$-expressing cells, in agreement with previous observations (Bennett, 2003; Wang *et al.*, 1996). Of note, we failed to detect any significant upregulation of p53 and $p21^{CIP1}$ in nevi (not shown). Irrespective of the mutational status of BRAF in the nevi, the percentage of $p16^{INK4a}$-positive cells and the intensity of staining per cell were heterogeneous, with a striking mosaic pattern of $p16^{INK4a}$ immunopositivity seen in most nevi (Figure 3a; Supplementary Figure 6; data not shown). Importantly, all melanocytes within these $p16^{INK4a}$-mosaic nevi, whether $p16^{INK4a}$-positive or $p16^{INK4a}$-negative, were growth-arrested (Figure 3a; Supplementary Figure 6). Indeed, the lack of proliferation of the nevus melanocytes was associated with SA-β-Gal positivity, rather than with $p16^{INK4a}$ immunoreactivity, thereby closely mimicking $BRAF^{E600}$-expressing melanocytes *in vitro*. As nevi are thought to be clonal (Robinson *et al.*, 1998), it is unlikely that a differential BRAF mutation pattern underlies the $p16^{INK4a}$ mosaicism. The observed $p16^{INK4a}$ mosaicism is in accordance with the occurrence of growth arrest in nevi of homozygous $p16^{INK4a}$-deficient 'Leiden' patients (Gruis *et al.*, 1995). However, the nevi of these patients are increased in number and size compared to those of $p16^{INK4a}$-proficient individuals. Together with the mosaic

p16^{INK4a} pattern seen in BRAF^{E600}-expressing, growth-arrested melanocytes *in vitro*, these observations suggest that p16^{INK4a} collaborates with other, yet to be identified factors in the establishment of long-term growth arrest of nevi. Whether p16^{INK4a} contributes to an irreversible arrest that, once established, no longer requires p16^{INK4a} (as has been suggested Beausejour *et al.*, 2003; Narita *et al.*, 2003) or is involved in other aspects of melanoma progression than OIS remains to be determined. In summary, human nevi and BRAF^{E600}-expressing melanocytes share three senescence-associated markers: stable growth arrest, heterogeneous induction of p16^{INK4a} and SA-β-Gal activity.

Although these features support OIS as the cause for cell cycle arrest of melanocytic nevi, in principle these senescence markers could all have been triggered also by telomere attrition, which might have resulted from the initial expansion of melanocytes. Indeed, telomere attrition has been previously proposed as a major factor involved in the cell cycle arrest of nevi (Bastian, 2003). This would be consistent with the clinical observation that in particular nevi that arise before birth (congenital nevi) can cover large areas of the body surface, in contrast to nevi acquired later in life, which usually are much smaller. To test this possibility, we performed telomere FISH (fluorescent in situ hybridization) on congenital nevus resections. As expected, melanoma metastases, stained as controls, displayed much less fluorescence than their surrounding stromal cells, indicating that this method is sufficiently sensitive to detect differences in telomere length *in situ* (Figure 4a and b; Supplementary Figure 7). In contrast, we did not observe any significant difference in telomere fluorescence

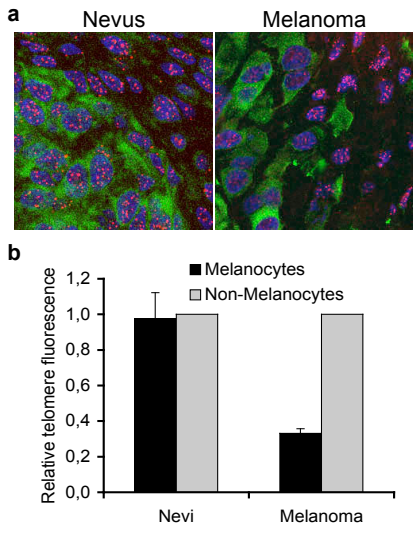

Figure 4 | ***No apparent telomere loss in nevi.*** **a |** Frozen sections of human nevi and melanomas were subjected to telomere FISH (in red). A representative sample is shown, out of 6 nevi analyzed (5 of which harbor the BRAF^{E600} mutation). For reference, sections were also stained with Melan A (detecting melanocytes in nevus and melanoma; in green) and DAPI (in blue). Note that in the nevus specimen, both the nevus cells (Melan-A positive) and the stromal compartment (Melan-A negative) are FISH-positive. In contrast, melanoma tumor cells, which conceivably have undergone telomere attrition, show much less telomere fluorescent signal than their surrounding stromal cells. The channel overlay is shown; individual channels are shown in Suppl. Fig. 7. **b |** Quantification of the data in a, plotted as relative telomere fluorescence in 6 nevi and 3 melanomas (both indicated as 'Melanocytes') compared to their respective stromal cells ('Non-melanocytes'), with standard deviations.

comparing congenital nevi to surrounding tissues, in agreement with previous observations made in acquired and Spitz nevi (Miracco *et al*., 2002). Although we cannot formally rule out that a single eroded telomere may have triggered the senescence response, one would not expect the other telomeres to remain apparently full length. These results, therefore, argue in favour of an active oncogene-driven senescence process, rather than senescence triggered by exhaustion of replicative potential resulting from gross telomere attrition.

Our observation that, initially, $BRAF^{E600}$ moderately stimulates melanocyte proliferation supports the hypothesis that it contributes to the initiating events of melanomagenesis. However, our results suggest that, both *in vitro* and *in vivo*, oncogenic BRAF signaling subsequently leads to a growth-inhibitory response, which is associated with the known classical hallmarks of senescence, i.e., stable proliferative arrest, an increase in $p16^{INK4a}$ and the induction of SA-β-Gal activity. Our results therefore provide support for a speculative model previously proposed by us and others (Bennett, 2003; Peeper and Mooi, 2002) in which $BRAF^{E600}$ cannot fully transform human melanocytes, but requires additional, cooperating events for tumour development. Supporting this view, zebrafish expressing a $BRAF^{E600}$ transgene develop 'fish-nevi', which require a p53-deficient background to progress to invasive melanomas (Patton *et al*., 2005). In sum, our observations provide evidence of oncogene-induced senescence (OIS) as a physiological mechanism in humans, limiting the progression of premalignant lesions.

MATERIALS AND METHODS

Plasmids

Gene transfer in normal melanocytes was achieved by lentiviral vectors. An aminoterminal HA-Tagged $BRAF^{V600E}$ was cloned under a CMV promoter in the lentiviral vector FG12 (also coding for GFP driven by a separate UbC promoter). The efficacy of gene transfer (green cells) was estimated to be approximately 90% by fluorescence microscopy. For retroviral infection, $BRAF^{E600}$ was cloned from the SK-MEL28 melanoma cell line into the pBabe-puro vector. The $p16^{INK4a}$ shRNA sequence corresponds to nucleotides 21-41 (GenBank accession number NM_000077). The BRAF shRNA sequence was previously described (Hingorani *et al*., 2003).

Cell culture, retroviral transduction, cell cycle analysis and proliferation curves

Normal human melanocytes were isolated from the epidermis of neonatal foreskins. Briefly, foreskins were incubated overnight in Trypsinization Solution (22.5 mM HEPES, 7.5 nM Glucose, 2.25 mM KCl, 100 mM NaCl, 0.75 mM Na_2HPO_4 and 0.17% trypsin). Dermis and epidermis were separated by scrapping. The epidermal compartment was incubated for 3-4 days in 254CF Medium (Cascade Biologicals) supplemented with 0.1 mM $CaCl_2$, 2% FBS and Keratinocyte Growth Supplement (Cascade Biologicals). Melanocytes were subsequently separated from keratinocytes by differential trypsinization. Purity of the melanocytic preparations was estimated by standard immunostaining for HMB-45. For long-term cultures, melanocytes were propagated in 254 CF Medium in the presence of 0.2 mM $CaCl_2$ and Melanocyte Growth Supplement (Cascade Biologicals). BJ cells expressing hTERT (in pBabeHygro)

were grown in DMEM/medium 199 (Gibco) in a 4:1 ratio supplemented with 15 % fetal bovine serum (FBS) (PAA Laboratories), 0.1 mM MEM non-essential amino-acids (Gibco), 2 mM glutamine (Gibco) and antibiotics. Lentiviral infections were performed using 293T cells as producers of viral supernatants. The Phoenix packaging cell line was used for the generation of ecotropic retroviruses, as described (Peeper *et al*., 2002). Amphotropic retroviruses encoding the ecotropic receptor were generated in HEK293 cells. All infections were carried out as described (Peeper *et al*., 2002). Bromodeoxyuridine (BrdU) labeling was carried out for 3 hours, in reduced growth factor conditions. Proliferation curves were performed as described (Serrano *et al*., 1997; Peeper *et al*., 2002).

Human tissue samples

Surgical resection specimens of congenital nevi were obtained from patients in their first year of life. Specimens were fresh frozen and stored for a period of 2 months to 2 years at –70°C prior to use.

SA-β-Galactosidase activity analysis

Tissues were fixed in 4% formaldehyde for 2 hours, washed with 0.2% NP-40, 0.1% Na-deoxycholate, 2 mM $MgCl_2$, 100 mM Na_2HPO_4 pH 6.0, and stained as described (Dimri *et al*., 1995); post-fixed overnight in 4% formaldehyde and embedded in paraffin. Cultured cells were stained as described (Dimri *et al*., 1995).

Immunohistochemistry and antibodies

Tissues were fixed in 4% formaldehyde overnight and embedded in paraffin. Paraffin sections were deparaffinized, rehydrated, incubated in 0.1mM sodium citrate pH 6.0, washed and incubated with peroxidase blocking reagent (#S2001, DAKO) and incubated with the primary antibodies: Ab-3 for MelanA (Molecular Probes); MIB-1 for Ki-67 (DAKO); Ab-4 (16P04) for $p16^{INK4a}$ (Molecular Probes). Secondary antibody used was PowerVision+ (DPVB+999HRP ImmunoLogic). Peroxidase activity was detected with Liquid DAB (K3468 DAKO). Antibodies used were: F-7 for BRAF (sc-5284, Santa Cruz), JC8 (MS-889, NeoMarkers) for $p16^{INK4a}$, C-22 for CDK4 (sc-260, Santa Cruz) and AC-74 for β-actin (A5316, Sigma) for Western blot and anti-trimethyl-Histone H3 (Lys9) (#07-442, Upstate) and 554070 (BD Pharmingen) for $p16^{INK4a}$ for immunofluorescence.

Telomere FISH

Tissue sections were prepared for fluorescence *in situ* hybridization (FISH) analysis with the 2'-deoxyoligonucleotide N3'®P5' phosphoramidate probe: 5'-$(CCCTAA)_3$-TAMRA-3', specific for telomeric sequences (red), and Melan A antibody Ab3 (Molecular Probes; green) and a common DNA staining dye DAPI (blue). Images of FITC, TAMRA, and DAPI fluorescence were acquired on a digital image microscopy system. The relative telomere length is proportional to the number of hybridized probes. For quantification, the fluorescence signal was determined (using ImageJ software) for all individual telomeres within 12 nuclei per specimen, both for melanocytes (within nevi and melanomas) and for cells within the stromal compartments of both types of lesions, for 6 independent nevi and 3 independent melanomas. All measurements have been corrected for background nuclear fluorescence.

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SUPPLEMENTARY MATERIALS AND METHODS

Tumourigenicity assay

Athymic nude mice where subcutaneously injected on both flanks with $1*10^6$ cells expressing hTERT, SV40 small tumor antigen, sh-p53, sh-$p16^{INK4a}$ and either $BRAF^{E600}$, RAS^{V12} or vector. Mice were sacrificed when tumors reached a diameter of 10mm. Experiments were approved by the Licensing Committee of the Netherlands Cancer Institute.

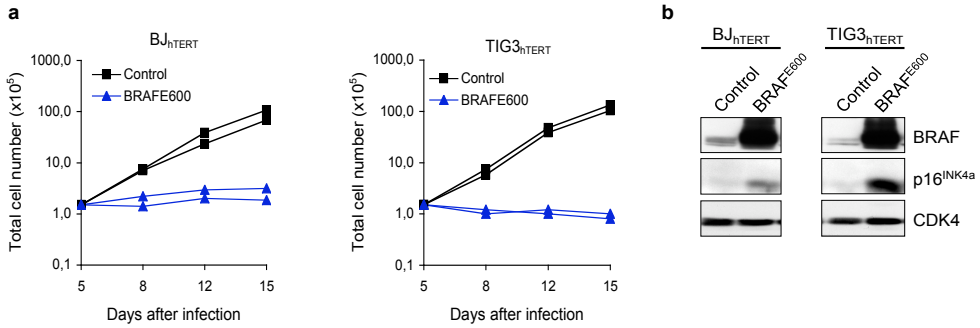
Cell culture

TIG3 cells and 'Leiden' fibroblasts, expressing hTERT, were grown in DMEM supplemented with 10% FBS, 2mM glutamine and antibiotics.

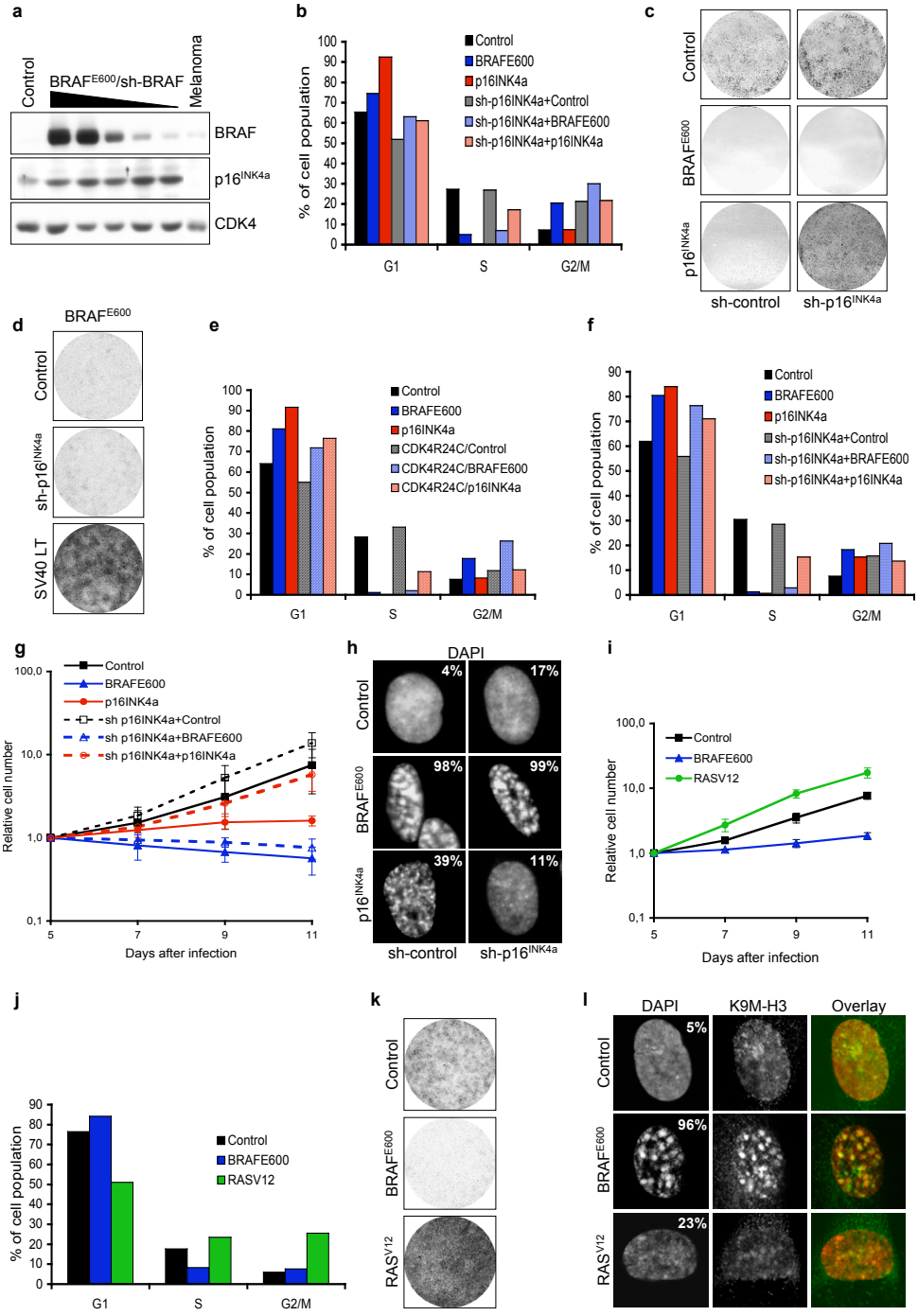
QRT-PCR and BRAF sequencing

QRT-PCR was performed on an ABI Prism 7000 Sequence Detection System (Applied Biosystems) using SYBR Green Master Mix (4309155, Applied Biosystems) according to the manufacturer's protocol. Primers used were: 5'-CCCTCGTGCTGATGCTACTGA and 5'-CATGACCTGGTCTTCTAGGAAGC for $p14^{ARF}$ (Accession Number NM_058195); and 5'-CTGCCCAACGCACCGA and 5'-CCATCATCATGACCTGGATCG for $p16^{INK4a}$ (Accession number NM_000077). BRAF sequencing was performed as previously described in Jarry, A. et al., Mol Cell Probes, 18, 349-52 (2004), using SYBR Green Master Mix (Applied Biosystems).

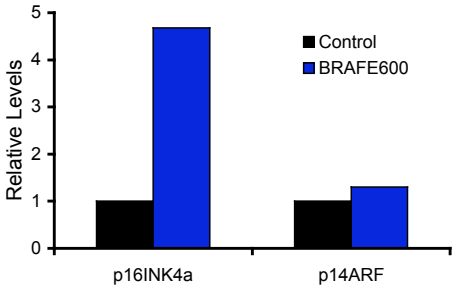
SUPPLEMENTARY FIGURES AND TABLE



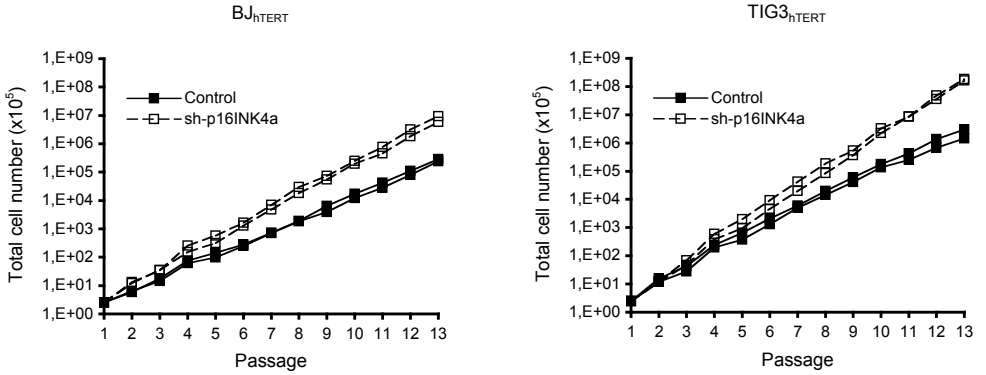
Supplementary Figure 1 | ***Ectopic overexpression of BRAF^{E600} causes a senescence-like cell cycle arrest.*** Human BJ_{hTERT} and $TIG3_{hTERT}$ fibroblasts were transduced with either control or $BRAF^{E600}$-encoding retrovirus and pharmacologically selected for stable genomic integration. **a |** Analysis by growth curves, performed in duplicate. **b |** Analysis by Western blotting with the indicated antibodies (CDK4 serves as a loading control).


a
Control
BRAF^E600/sh-BRAF
Melanoma
BRAF
p16^INK4a
CDK4
b
% of cell population
Control
BRAFE600
p16INK4a
sh-p16INK4a+Control
sh-p16INK4a+BRAFE600
sh-p16INK4a+p16INK4a
G1
S
G2/M
c
Control
BRAF^E600
p16^INK4a
sh-control
sh-p16^INK4a
d
BRAF^E600
Control
sh-p16^INK4a
SV40 LT
e
% of cell population
Control
BRAFE600
p16INK4a
CDK4R24C/Control
CDK4R24C/BRAFE600
CDK4R24C/p16INK4a
G1
S
G2/M
f
% of cell population
Control
BRAFE600
p16INK4a
sh-p16INK4a+Control
sh-p16INK4a+BRAFE600
sh-p16INK4a+p16INK4a
G1
S
G2/M
g
Relative cell number
Control
BRAFE600
p16INK4a
sh p16INK4a+Control
sh p16INK4a+BRAFE600
sh p16INK4a+p16INK4a
Days after infection
h
DAPI
Control
BRAF^E600
p16^INK4a
4%
17%
98%
99%
39%
11%
sh-control
sh-p16^INK4a
i
Relative cell number
Control
BRAFE600
RASV12
Days after infection
j
% of cell population
Control
BRAFE600
RASV12
G1
S
G2/M
k
Control
BRAF^E600
RAS^V12
l
DAPI
K9M-H3
Overlay
Control
BRAF^E600
RAS^V12
5%
96%
23%

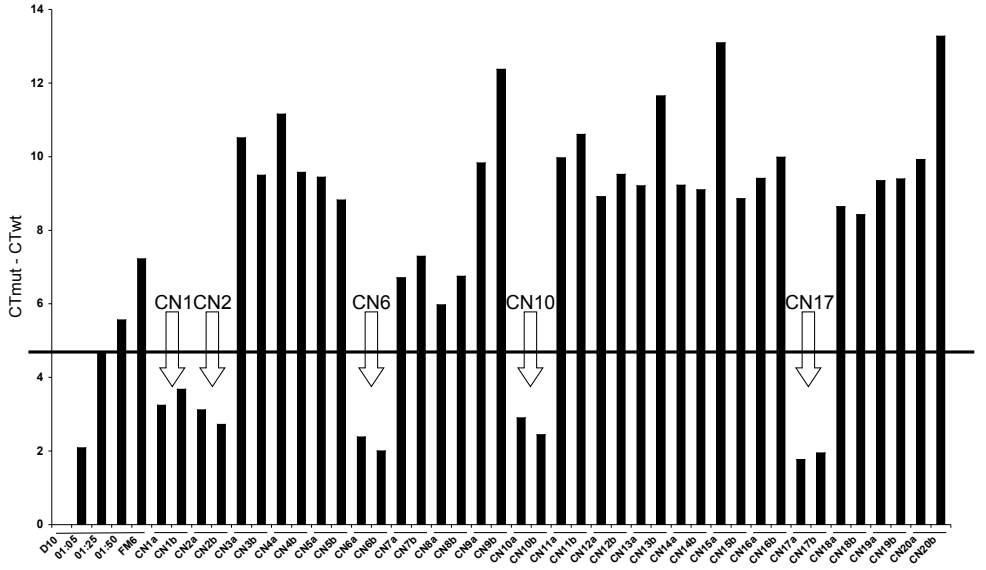
◄ **Supplementary Figure 2 |** ***Physiologic levels of BRAF^{E600} induce senescence in a p16^{INK4A}-independent manner.*** **a-c |** Human BJ_{hTERT} cells were transduced with a mixture of a retrovirus encoding a $BRAF^{E600}$ expression cassette, and an increasing amount of a retrovirus encoding a short hairpin (sh) RNA targeting both endogenous wild type and ectopic $BRAF^{E600}$, followed by pharmacologic selection for cells that had stably integrated both the expression cassette and the shRNA (the sh-BRAF alone did not affect proliferative potential; not shown). This yielded progressively decreasing $BRAF^{E600}$ levels, comparable to those seen in melanoma cells, as analyzed by Western blotting (**a**; CDK4 serves as a loading control). **b |** BrdU incorporation and DNA-content analysis (PI) of BJ cells described in Fig. 2 a-c (the result shown is representative of 2-4 independent experiments). **c |** Analysis by a colony formation assay (stained 11 days post-infection). **d |** Bypass of $BRAF^{E600}$-induced senescence by co-expression of SV40 large tumor antigen (SV40 LT), as measured by a colony formation assay (stained 16 days p.i.). **e |** BrdU incorporation and DNA-content analysis (PI) of BJ cells infected with retrovirus encoding $p16^{INK4a}$-insensitive $CDK4^{R24C}$ and a retrovirus mixture driving low levels of $BRAF^{E600}$. **f-h |** Primary human $TIG3_{hTERT}$ fibroblasts expressing hTERT were transduced as in **b**. **f |** Analysis was performed by BrdU incorporation and DNA-content analysis (PI; the result shown is representative of 2-4 independent experiments). **g |** Analysis by proliferation curves (result is from three independent experiments performed in duplicate, shown with standard deviations). **h |** DAPI staining to detect SAHF (percentage of SAHF-positive cells is indicated in insert). **i-l |** Primary 'Leiden' fibroblasts expressing hTERT were transduced as in **b**. As a control, cells were transduced with RAS^{V12}-encoding retrovirus. **i**, Analysis by proliferation curves (result is from three independent experiments performed in duplicate, shown with standard deviations). **j |** BrdU incorporation and DNA-content analysis (PI; the result shown is representative of 3 independent experiments). **k |** Analysis by a colony formation assay (stained 14 days post-infection.). **l |** Analysis by DAPI staining to detect SAHF (percentage of SAHF-positive cells is indicated in insert) and immunofluorescence for K9M-H3. Note that $BRAF^{E600}$-expressing cells underwent cell cycle arrest. In contrast, these $p16^{INK4a}$-deficient human cells were completely resistant to RAS^{V12}-induced senescence, consistent with previous results (Brookes, S. et al., EMBO J, 21, 2936-45 (2002)) and indicative of differential activation of tumor suppressor signalling by $BRAF^{E600}$ and RAS^{V12}.



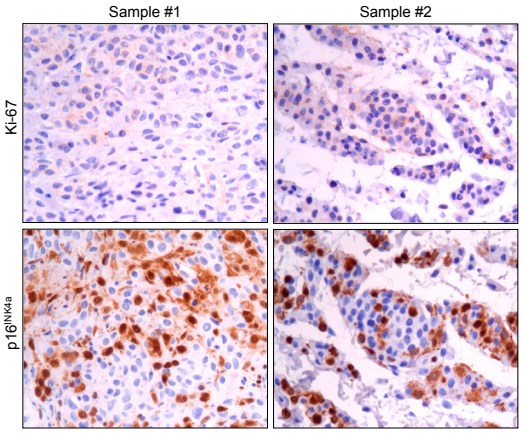
Supplementary Figure 3 | ***BRAF^{E600} fails to induce p14^{ARF}.*** Human BJh_{TERT} cells were transduced with a mixture of retrovirus driving low levels of $BRAF^{E600}$, as described for Suppl. Fig. 2. Analysis was performed by QRT-PCR for *$p14^{ARF}$* and *$p16^{INK4a}$*.



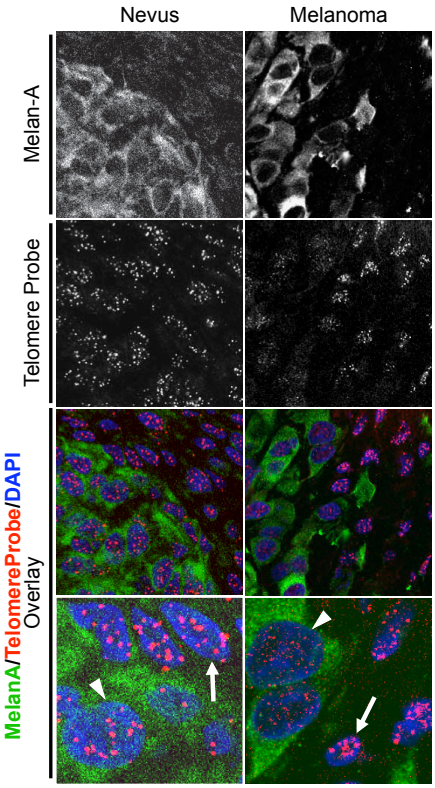
Supplementary Figure 4 | ***Sustained knockdown of p16^{INK4A} causes an increase in cellular proliferation rate.*** Primary human BJ_{hTERT} and $TIG3_{hTERT}$ cells were transduced with retrovirus encoding $p16^{INK4a}$ shRNA (co-expressing a GFP cassette), selected for stable genomic integration by FACSorting and subjected to a 3T3 protocol.



Supplementary Figure 5 | ***BRAF^{E600} mutational analysis in human congenital nevi.*** Y-axis represents the difference in PCR cycles needed to reach a threshold signal (CT) between the mutant-specific (CT_{mut}) and wt-specific (CT_{wt}) primer pairs. D10, a melanoma cell line with the a $BRAF^{E600}$ allele and FM6, a melanoma cell line with two wt BRAF alleles were used as controls. Decreasing the D10:FM6 gDNA ratio (i.e. decreasing amounts of $BRAF^{E600}$ template) result in increasing number of cycles needed to reach threshold signal for the mutant specific primer pair (CT_{mut}), and therefore in increasing CTmut-Ctwt difference. Black cut-off line represents the cycle-difference threshold [(CT_{mut})–(CT_{wt})] below which samples are considered to have a $BRAF^{E600}$ allele (samples shown by white arrows). Each sample was analyzed in duplicate (a, b) and in at least two independent reactions.



Supplementary Figure 6 | ***Mosaic pattern of p16^{INK4A}-positivity in nevi.*** Paraffin-embedded sections of 2 representative human nevi were subjected to immunohistochemistry with the indicated antibodies. MIB1 (brown) recognizes the proliferation marker Ki-67 and p16^{INK4a} antibody detects p16^{INK4a}. Note that both nevi contain melanocytes abundantly expressing p16^{INK4a} surrounded, in seemingly irregular patterns, by melanocytes devoid of p16^{INK4a} immunoreactivity. In individual nevi, the percentage of p16^{INK4a}-negative melanocytes ranged from <10% to >90%.



Supplementary Figure 7 | ***No apparent telomere loss in nevi.*** Frozen sections of human nevi and melanomas were subjected to telomere FISH (in red). A representative sample is shown, out of 6 nevi analyzed (5 of which harbour the BRAF^{E600} mutation). For reference, sections were also stained with Melan A (detecting melanocytes in nevus and melanoma; in green) and DAPI (in blue). The bottom row represents a zoom of the overlay. Arrowheads point at melanocytes (both in the nevus and the melanoma), arrows point at neighbouring stromal cells. Note that in the nevus specimen, both the nevus cells (Melan-A positive) and the stromal compartment (Melan-A negative) are FISH-positive. In contrast, melanoma tumor cells, which conceivably have undergone telomere attrition, show much less telomere fluorescent signal than their surrounding stromal cells.

Tumor induction after 8 weeks

Cells	Exp #1	Exp #2
Control	0/8	0/8
BRAF^{E600}	7/8	8/8
RAS^{V12}	8/8	7/8

Supplementary Table 1 | BJ$_{hTERT}$ cells expressing SV40 small tumor antigen, sh-p53, sh-p16^{INK4A}, and either BRAF^{E600} or RAS^{V12} were injected subcutaneously into both flanks of nude mice and tumor formation was assessed 8 weeks later.